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Electrocardiographic, Heart-Rate and Subjective Responses to Prolonged Gravitational Stress and their Relation to some Dimensional and Functional Parameters of the Circulatory System

By

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Abstract

BJURSTEDT, H., L.-E. HANSSON and G. STRÖM. Electrocardiographic, heart-rate and subjective responses to prolonged gravitational stress and their relation to some dimensional and functional parameters of the circulatory system. *Acta physiol. scand.* 1959. *47*. 97—108. — Heart-rate and ECG (including 5 precordial leads) responses were studied in 16 healthy subjects during up to 6 min exposure to 3—$3^1/_2$ times the force of gravity in a human centrifuge. The subjective *g* tolerance (resistance to dimming or loss of vision) was determined in relation to various circulatory functional and dimensional parameters. The ECG did not show any remarkable alterations during or after the runs. Subjective *g* tolerance was correlated to orthostatic heart-rate (produced by normal gravity) but not to heart-rate response under increased gravitational stress, physical working capacity, heart volume in the horizontal or vertical body position, or total hemoglobin.

The present report is ultimately concerned with the problem of the prediction of individual tolerance to prolonged gravitational stress in the head-to-seat direction ("positive radial acceleration") under flying conditions. A

number of circulatory responses to acceleration have been investigated under laboratory conditions, the accelerative force being applied by the use of a large centrifuge.

The first part of our report describes the ECG changes which occur during 6 min exposure to 3—3½ *g*. The exposure was continued up to the moment when grayout occurred (grayout level) and seemed to develop into complete loss of vision (blackout). This was done in order to estimate the risk of serious arrhythmia which has been elsewhere reported to occur during positive acceleration of short (GAUER 1950) or moderately long duration (ZUIDEMA *et al.* 1956).

The second part describes the heart-rate response during acceleration in relation to the degree of stress. The subjective visual symptoms of trained subjects were regarded as a sufficiently valid index of bodily resistance to accelerative force ("subjective *g* tolerance").

The third part describes the relationship between the grayout level and certain dimensional and functional parameters of the circulatory system, such as heart and blood volumes and the reactions during standardized orthostatic and work tests.

Subjects, Methods and Procedure

Subjects. Experiments were performed on 13 male and 3 female healthy subjects. All were familiar with subjective stresses experienced during runs in the centrifuge. Therefore they did not show any signs of apprehension or fear prior to or during the runs. Their heart rates immediately before the runs were not high. The body statures of the subjects were ordinary (see Table III). *g*-suits were not used, and the subjects were instructed to abstain from straining or other anti-*g* maneuvers.

Centrifuge. The main features of the centrifuge have been described elsewhere (GÖTZLINGER and HELSING 1955, BJURSTEDT 1957). The cabin is suspended at one end of the double-arm superstructure of the centrifuge, 24 ft from the center of rotation. As the centrifuge is started, the cabin swings out so that, when a constant speed has been attained, its floor remains perpendicular to the direction of the resultant force. In the present series of experiments the resultant force was not higher than 3½ *g*. All runs were controlled so that the *g* pattern as a function of time had the shape of a single plateau. The time used for starting and stopping the centrifuge amounted to 5—10 sec. The subject's face as well as the upper part of his torso was watched continuously by means of a closed-circuit television monitor.

Subjective g tolerance. As a criterion of the tolerance to *g* of the individual the subjective sensation of marked dimming of vision was used. Other current methods which use central or peripheral light sources in combination with signalling systems do not at present seem to offer any significant advantages for the estimation of the bodily reaction and resistance to positive accelerative force.

Laboratory Methods. The total amount of hemoglobin (THb) was determined by the alveolar CO method (SJÖSTRAND 1948) with minor modifications (CARLSTEN *et al.* 1954, see also WIKLANDER 1956). The hemoglobin (Hb) concentration was determined spectrophotometrically as oxyhemoglobin in alkaline solution. The total blood volume was calculated from the THb and Hb values. The total heart volume was

determined in the horizontal (prone) and standing body positions by two-plane roentgenograms and calculated according to LILJESTRAND *et al.* (1939), as modified by JONSELL (1939). An orthostatic test was carried out, consisting of passive standing against a wall for eight minutes with recordings of the heart rate and the ECG at the end of the period. The physical working capacity (PWC) was determined by a standardized work test (SJÖSTRAND 1947, WAHLUND 1948), using 6 min periods of stepwise increasing loads (with registration of heart rate, respiratory rate and ECG), and expressed as work load at a heart rate of 170 beats/min under steady-state conditions. The PWC determined by this test is an index of the circulatory functional capacity, being mainly dependent on cardiac stroke volume and peripheral circulatory adaptation. A further description and discussion of the above-mentioned methods, as well as of the evaluation of the results obtained thereby, can be found in a recent report by HOLMGREN *et al.* (1957).

The ECG was recorded by a 4-channel direct-writing apparatus (Mingograf 42, Elema, Stockholm); leads I, II, III, aVR, aVL, aVF, CR1-2-4-5-7 and V1-2-4-5-7 were taken, before the run, with the subject being first in the recumbent position and then sitting in the cabin. During the run, the ECG was continuously recorded, leads I, II, III and CR1, or CR2-4-5-7 being used alternately.

The heart rate was either recorded directly on photographic paper by means of an instantaneous cardiotachometer (STURM and WOOD 1947) or read from the ECG. The arterial blood pressure was recorded at rest and in the orthostatic test, by the auscultatory cuff method, diastolic pressure being read at the disappearance of the KOROTKOW sounds.

Results

ECG reaction. The resting ECG:s were all normal. The ECG was recorded continuously in 12 male subjects at 3 *g* and in 8 of them also at $3^1/_2$ *g* during runs of up to 6 min duration (subjects of Table III). No arrhythmia was observed before, during or after the runs in any of the subjects. There were generally signs of slight to moderate cardiac displacement, the heart being rotated on its antero-posterior axis to a more vertical position (Fig. 1). Simultaneously with the increase of heart rate during a run, slight to moderate depression of the S—T segments and flattening of the T waves occurred together with the appearance of a moderate positive afterpotential (described by SJÖSTRAND 1951). These changes of S—T and T were of the physiological type (Fig. 1, upper part), usually observed in tachycardia (SJÖSTRAND 1950), and not suggestive of coronary insufficiency. They quickly subsided after the end of a run. In one of the 12 subjects (EK, Table III), the ECG reaction during the work test was abnormal, since ventricular extrasystoles occurred at the highest load (Fig. 1, lower part). This young man was, and earlier had been, subjectively quite healthy, and repeated clinical investigations (including *i. a.* roentgenography of the heart, ECG at rest and during work, and antistreptolysin and antistaphylolysin activities in blood) failed to reveal signs of infectious disease or functional myocardial disturbance. The abnormal ECG reaction in the first work test may therefore have been due to a high myocardial excitability of non-pathological nature; in any case it is especially

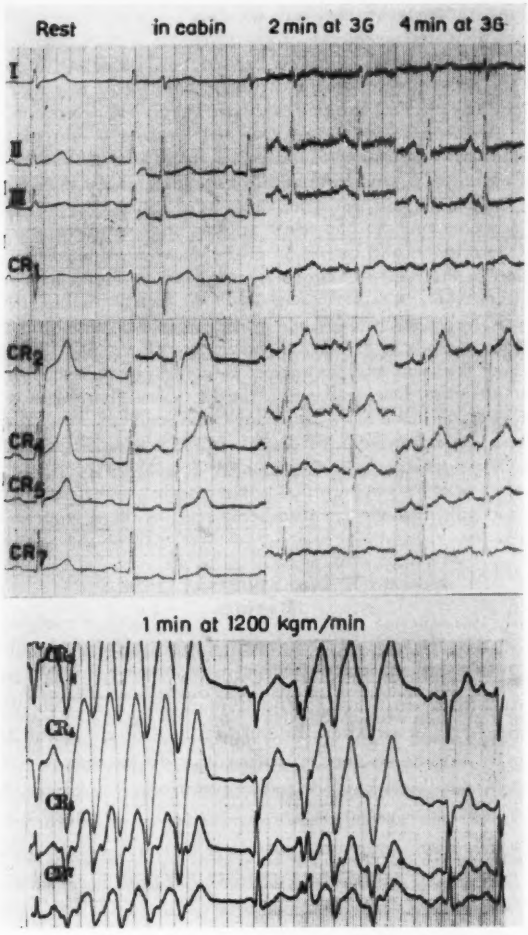


Fig. 1. Upper part; typical ECG response to prolonged gravitational stress (3 *g* positive acceleration, subject *OL* in Tab. 3).

Lower part; serial ventricular extrasystoles occurring in subject *EK* during work test.

interesting that even in this subject no arrhythmia occurred during acceleration.

Heart-rate reaction. The heart-rate reaction during a run at 3 *g* of up to 6 min duration was recorded on several occasions in each of 16 subjects (Table I, Table III). The typical reaction consisted of a rapid rise up to a new constant

Table I Heart rates after 1 min of gravitational stress at 3 g, and further changes after 2, 3, 4, and 5 min of continued stress in relation to the one-minute value. Thirteen male and 3 female subjects between 19 and 37 years of age. Each subject was run on 1—10 occasions (average 5). All heart-rate values were first averaged for each subject. Number of subjects, mean values and ranges are given.

	Heart rate	Heart-rate change in relation to one-minute value			
	1 min	1—2 min	1—3 min	1—4 min	1—5 min
n	16	16	13	12	8
M.....	127	+ 3	+ 5	+ 4	+ 8
Range.....	106/161	+13/−15	+21/−5	+29/−10	+28/−15

level, sometimes with an initial transitory overshoot (Fig. 2, upper part). A less common reaction was also observed where the heart rate continuously rose during the run (Fig. 2, lower part). The acceleration from 1 *g* to 3 *g* in the centrifuge usually took about 5 sec; the heart rate usually reached the new level within 30—60 sec. The average heart rate at 3 *g* was 127 beats/min (Table I), the inter-individual range being rather large (106—161). In this series of observations there was no obvious correlation between level of heart rate and subjective *g* tolerance.

When, in the course of the investigation, runs were made with a subject on several occasions, there was a tendency for the heart-rate level during *g* stress to become lower (by about 10 beats/min), mainly appearing between the first and the third individual runs (Table II), but the change is not statistically significant. There was a relatively large random variation of the heart-rate response in consecutive runs for each subject (Table II).

In the 12 subjects shown in Table III, the average heart rate after 2 min at 3 *g* was the same in subjects with high and medium subjective *g* tolerance, but slightly higher, although not statistically different, in subjects with low *g* tolerance (Table IV).

Circulatory dimensions and function. The physical working capacity and its two dimensional correlates, total hemoglobin and heart volume, were interrelated within the normal limits (Fig. 3) in the 12 specially studied subjects. In this respect there was no apparent difference between subjects with different degrees of *g* tolerance (Table IV, Fig. 3). The body dimensions (Table IV) were also similar in the three groups of subjects.

The orthostatic reactions during 8 min standing were measured as heart-rate increase, arterial blood pressure decrease, heart-volume decrease, and as the relationship between orthostatic heart rate and the Height/$(THb)^{1/3}$ ratio (Fig. 3, Table IV). This ratio constitutes an anthropometric index, which normally is correlated to the orthostatic heart rate (HOLMGREN *et al.* 1957).

Table II Average change in heart rate (at 3 g after 1 min) from the first to

Run no.	2	3	4	5
n	13	11	10	9
M	− 4	−13	− 7	−12
Range	+25/−35	+30/−30	+35/−25	+35/−40

Subjects with a high *g* tolerance showed a lower pulse rate, both in absolute terms and in relation to the Height/$(THb)^{1/3}$ ratio, a slightly higher diastolic blood pressure, and a smaller heart-volume decrease in standing than did subjects with a low *g* tolerance.

Discussion

The ECG response to prolonged accelerative stress was not remarkable in the present series of experiments. Our observations differ from the earlier findings of GAUER (1950), and especially of ZUIDEMA *et al.* (1956), who found serious arrhythmia during prolonged exposures (*e. g.* auricular fibrillation in 2, and supraventricular or ventricular extrasystoles in 2 out of 5 subjects), prominent S—T and T changes, and subjective heart symptoms (*e. g.* substernal

Table III Individual values in 12 male subjects. Gr = grayout. The

Subj.	Age, years	Weight, kg	Height, cm	THb, g	Hb conc., g/100 ml	Blood vol., l	Heart volume, ml			PWC, kgm/min
							standing	lying	increase lying	
SV	22	68	186	873	14.1	6.2	730	800	70	1,300
FL	26	70	178	731	15.0	4.9	615	680	65	900
PE	19	67	182	689	13.5	5.1	740	860	120	1,000
AN	23	67	175	725	16.6	4.4	680	770	90	1,000
FÄ	19	60	166	637	15.8	4.0	620	800	180	1,000
OL	32	72	188	700	14.8	4.7	650	720	70	900
EK	25	73	192	831	14.3	5.8	700	730	30	1,000
BA	33	71	180	613	13.8	4.4	710	870	160	1,000
HU	37	78	176	590	12.3	4.8	730	820	90	900
ÖS	25	64	175	643	13.3	4.8	800	780	70	900
DR	25	80	187	926	14.8	6.3	750	940	190	1,300
NY	25	68	184	825	15.0	5.5	630	900	270	900

each of the consecutive runs. Number of subjects, mean values and ranges are given.

6	7	8	9	10
6 −10 +35/−40	5 −12 +15/−35	5 −19 +20/−60	3 −22 +30/−55	2 −20 +15/−55

pain). We are at a loss to explain the conflicting results in the different series, unless the earlier investigation did not deal exclusively with healthy subjects or refer to substantially higher *g* levels (it has not been possible to draw definite conclusions from the above-mentioned reports in these respects). At any rate, our observations on a healthy material are comforting, since not even a refined ECG technique revealed any signs of coronary insufficiency or of other functional disturbances that would necessitate special caution from the cardiovascular point of view. Definite risks cannot be excluded with *g* loadings greater than $3^1/_2$ *g* or with exposures longer than 6 minutes, but the present series does not indicate which types of disturbances might be most likely to occur under such conditions.

In the following discussion reference is made to the term "subjective *g* tolerance", used here to denote the capability ("high", "medium", and "low") of the body to mobilize compensatory mechanisms for the maintenance

subjects are arranged in rising order of heart rate after 2 min at 3 g.

Heart rate, beats/min						Subjective *g* tolerance	Heart rate, beats/min		Art. blood press., mm Hg	
3 *g*			$3^1/_2$ *g*				lying	standing	lying	standing
2 min	4 min	6 min	2 min	4 min	6 min					
82	83	82	106	112	Gr	medium	56	60	120/80	120/80
102	100	110	86	86	86	high	60	68	135/90	120/90
106	106	Gr	130	Gr	—	rel. low	84	88	135/70	130/80
108	104	104	—	—	—	medium	64	94	125/70	105/75
108	110	108	—	—	—	medium	64	96	120/95	120/95
110	110	110	84	90	88	high	52	68	120/80	120/85
114	118	116	—	—	—	medium	88	92	125/65	115/80
120	120	—	112	112	102	high	60	80	115/75	110/80
122	124	124	140	140	Gr	medium	52	92	115/70	110/80
126	130	130	160	172	Gr	medium	76	88	130/80	130/80
132	Gr	—	—	—	—	low	76	98	125/80	125/80
156	156	Gr	184	Gr	—	rel. low	68	104	120/65	120/70

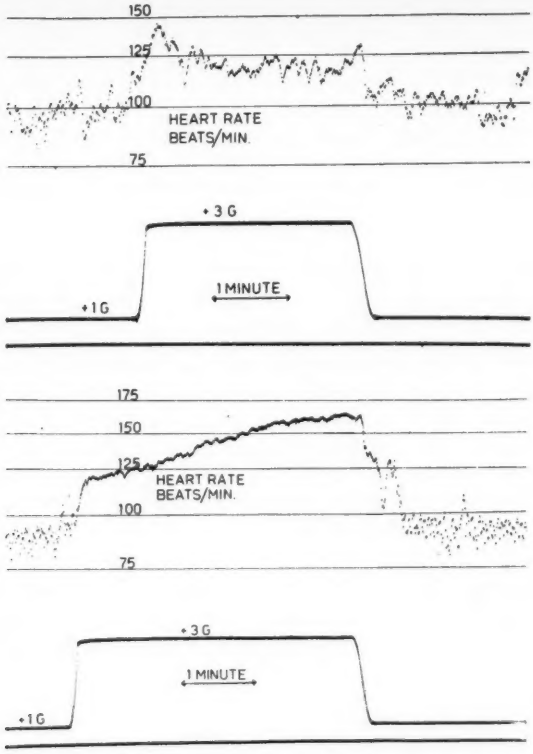


Fig. 2. Upper part; typical heart-rate response to prolonged gravitational stress (3 *g* positive acceleration).

Lower part; atypical heart-rate response.

of vision and consciousness in an individual exposed to prolonged *g* loadings in the head-to-feet direction. The subjective *g* tolerance may be regarded, therefore, as a convenient measure, although not of high precision, of the overall efficiency of the compensatory mechanisms. Locally reduced blood flow and a resulting anoxia in the retina and the brain is generally assumed to be the ultimate cause of the dimming or complete loss of vision and consciousness that may result from such stresses (reviewed by DUANE 1953).

The physiological response of a subject placed in a high gravitational field may make certain mechanisms clear which would otherwise be difficult to demonstrate. Thus, the human centrifuge offers unique possibilities to produce massive changes in the hydrostatic pressures exerted by the blood in different vascular regions. For instance, results obtained from centrifuge experiments

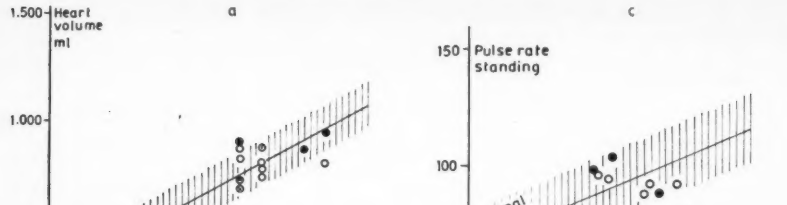
Table IV Mean values for three groups of male subjects (Tab. III) with high, medium and low tolerance, respectively, to prolonged g stress.

Subjective *g* tolerance	High	Medium	Low
Grayout latency period at 3 *g*, min	6	6	3
Grayout latency period at 3 $^1/_2$ *g*, min	6	4	2
Number of subjects	3	6	3
Age, years	30	25	23
Weight, kg	71	68	72
Height, cm	182	178	184
Total hemoglobin, g	681	717	813
Hemoglobin conc., g/100 ml	14.5	14.4	14.4
Blood volume, l	4.7	5.0	5.6
Heart volume in prone body position, ml	756	806	899
Physical working capacity, kgm/min at pulse 170	933	1,016	1,133
Heart volume decrease on standing, ml	98	88	193
Arterial blood pressure, resting, mm Hg	123/82	122/77	127/72
Arterial blood pressure, standing 8 min, mm Hg	115/85	117/82	125/77
Heart rate, resting	57	67	76
Heart rate, standing 8 min	72	87	97
Heart rate, 3 *g*, 2 min	111	110	131
Heart rate, 3 $^1/_2$ *g*, 2 min	94	(135)	(152)

may logically be applied to physiological events occurring during the more familiar stresses enduced by gravity in the erect position. The "stimulus" is exaggerated in the former case, and tendencies of various physiological responses accordingly more clearly demonstrable. Moreover, the relatively long-lasting gravitational stresses, which were applied in the present investigation, make it both possible and desirable to compare the results obtained with those from other types of stresses, in which the time factor is of the same order of magnitude. Consequently, attempts were made to correlate physiological responses observed during the centrifuge runs on one hand, with responses obtained in standardized orthostatic and work tests on the other.

Theoretically, the *g* tolerance should be expected to be dependent both on dimensional factors, such as body height and perhaps also blood volume, and on regulative factors, such as vasomotor adjustments of resistance and capacity vessels during the run. Empirically, other factors have been found to influence *g* tolerance, such as hypoglycemia and hyperventilation (BRENT *et al.* 1957). By using trained subjects and standardized conditions, as in the present investigation, the importance of hyperventilation and hypoglycemia is probably lessened. Excessive hyperventilation could be directly observed only in one subject (DR Table III), on the television screen. It is interesting that this subject showed the lowest *g* tolerance of all.

There was a lack of intra-individual correlation between the heart-rate



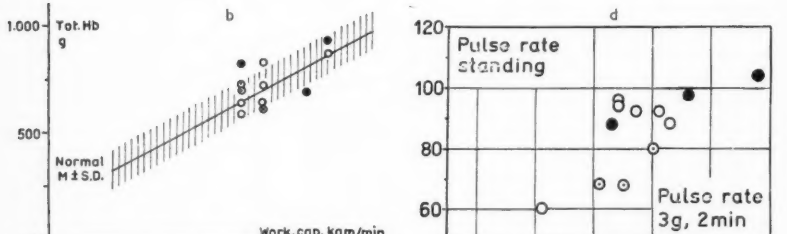


Fig. 3. The subjective g tolerance of the 12 subjects in Tab. III in relation to the normal relationships between (a) heart volume and physical working capacity, (b) total hemoglobin and physical working capacity, and (c) orthostatic heart rate and the Height/$(THb)^{1/3}$ ratio. The heart rate after 8 min passive standing is also shown in relation to the heart rate after 2 min at 3 g (d).

Filled circles: low subjective g tolerance; open circles: medium; dotted circles: high.

response during acceleration and the orthostatic heart-rate. This may seem surprising, since cardio-acceleration must conceivably result from a common stimulus in the two cases (only stronger in the centrifuge case), the time factor also being similar. There is little reason to assume that emotional factors played any significant role for the heart-rate response in either case. However, strong gravitational fields may cause specific effects on various regulative mechanisms, which may be triggered by exposure to 3 g, but not by normal gravity. Thus, hyperventilation may, by way of hypocapnia (alkalosis), modify the heart-rate response. The same holds true for hypoxemia, whether generalized or localized in the carotid bodies. These effects have been observed in anesthetized dogs exposed to moderate g loadings in the head-to-tail direction (Barr, Bjurstedt and Coleridge 1959), and may to some extent have been present in some of our subjects, thereby modifying their heart-rate response.

The heart-rate response to prolonged g stress was not closely related to g

tolerance; *i. e.* grayout and blackout occurred at different heart-rate levels in different individuals. This may imply that cardioaccelerator tone, and the vasoconstrictor tone in the lower half of the body do not closely change together. It has indeed already been suggested (COHEN *et al.* 1957) that different individual patterns of vasomotor and adrenal medullary reactions to *g* stress can be found.

A correlation with subjective *g* tolerance seemed to be apparent in the relationship between orthostatic pulse rate and the Height/$(THb)^{1/3}$ ratio. This observation suggests that a further investigation is needed, where a larger variation of subjective *g* tolerance should be investigated to demonstrate the possible predictive value of the relationship. It is of special interest when the significance of physical training for *g* tolerance is considered, since physical training produces a marked decrease of the orthostatic heart rate in relation to the above-mentioned circulatory/anthropometric relationship (HOLMGREN *et al.* 1957).[1]

Summary

1. The circulatory response to prolonged gravitational stress has been investigated and correlated to certain parameters of the circulatory dimensions and functional capacity. Sixteen subjects were exposed, in conventional sitting position, to positive (head-to-seat), radial acceleration in a centrifuge cabin for periods of up to 6 min, the accelerative force being kept constant at 3 *g* or $3^1/_2$ *g*. Each subject was tested repeatedly (from 3 to 12 times) on different days. Heart rate and ECG (including 5 precordial leads) were continuously recorded before, during and after the runs. Twelve of the subjects were examined for total amount of hemoglobin and blood volume, roentgenological heart volume in the prone and standing positions, and for heart rate and ECG responses to standardized orthostatic and work tests.

2. The ECG did not show any remarkable alterations during or after the runs, not even during greyout.

3. The heart rate rose within $^1/_2$—1 min to a new and higher level, then usually stayed relatively constant during the remainder of the runs, even during greyout. In a few subjects, however, the heart rate rose continuously throughout the runs. The average heart rate level during the periods of exposure to 3 *g* was 127/min in 16 subjects, range 106—161.

4. The orthostatic heart rate, especially when related to an anthropometric/circulatory index, appeared to be correlated to subjective *g* tolerance. There was, on the other hand, no clear correlation between subjective *g* tolerance and heart rate under increased gravitational stress, physical working capacity, heart volume in the horizontal or vertical body position, or total amount of hemoglobin.

[1] Note added in proof: In a recent report [M. K. Browne and J. T. Fitzsimons: Electrocardiographic changes during positive acceleration, Brit. Heart J. 1959. *21*. 23—30] no electrocardiographic abnormalities were observed to occur during shortlasting *g* stress in the centrifuge. This is in conformity with the present observations.

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On the Biological Assay of Cholecystokinin and Its Dosage in Cholecystography

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Abstract

E. JORPES, V. MUTT and L. OLBE. On the biological assay of cholecystokinin and its dosage in cholecystography. *Acta physiol. scand.* 1959. *47.* 109—114. — The methods applied in assaying the biological activity of cholecystokinin are reviewed. The authors recommend the technique of Ivy as described in detail by IVY and JANECEK 1959. One Ivy dog unit of cholecystokinin per kg body weight is recommended as a suitable dose to be given intravenously in man in connection with cholecystographies. No better response will be obtained with larger doses. This calculated dose agrees well with the dose found empirically to be clinically sufficient as applied in about 2,000 cholecystographies performed in different Swedish clinics.

The biological assay

In their first paper on cholecystokinin (CCK), the gallbladder-contracting hormone, IVY and OLDBERG (1928 a, b) assayed the strength of the preparations in anesthetized dogs by registering the increase in pressure in the cannulated gallbladder *in situ*, with the cystic duct clamped. In 1948 SNAPE, FRIEDMAN and THOMAS recorded the increase in pressure in the cannulated common bile duct of unanesthetized dogs equipped with permanent fistulas of the duodenum and of the stomach. The dogs could be used repeatedly during the course of up to 2—3 years.

A technique of using anesthetized dogs for simultaneous assay of both secretin and

CCK was in 1949 elaborated by GERSHBEIN, WANG and IVY. Under pentobarbital anesthesia the main pancreatic duct was cannulated and a grooved metal cannula secured in the gallbladder after clamping or ligating the cystic duct. For the secretin assay the juice output was registered on a drop recorder. The increase in pressure in the gallbladder was registered as a measure of the CCK activity. Simultaneously blood pressure recordings were made to test the absence of vasodilatin in the samples. After each addition of pentobarbital sufficient time was allowed to elapse before a new dose was administered. For both entities, secretin and CCK, either the S-shaped dose-response curves of the standard and the unknown could be used directly or the log dose-response curves, which are linear at a certain distance from the respective maxima. Usually a dose of 0.5—4 mg of CCK was injected into the dogs, but even 0.2—0.6 mg of one preparation showed good activity in all the four dogs used for the assay. The individual variation between the animals was very large, but when an unknown sample was tested against a standard sample in several dogs the recordings from the different animals indicated the strength of the sample with great accuracy as well in the secretin as in the CCK-tests. Thus 6 samples of secretin were tested against a standard in from 7 to 11 dogs each and 5 samples of CCK — likewise against a standard sample in from 4 to 10 dogs each with an almost identical unitage of secretin and of CCK respectively found in the different dogs.

HAVERMARK and HULTMAN used in 1952 a method of recording the contractions of the gallbladder of the cat *in situ*. They referred to previous techniques of similar nature performed on frogs (SEAGER 1939) and on cats and dogs (IVY and OLDBERG 1928 a, b, IVY *et al.* 1929, IVY *et al.* 1930, IVY, DREWYER and ORNDOFF 1930). A cannula was introduced through the papilla of Vater into the common bile duct. The hepatic ducts were ligated and the contractions of the gallbladder recorded on a manometer in mm of water. A dose response curve was constructed. The method was at one time used for the standardization of our CCK preparations.

As an adjunct to the dog procedure, GERSHBEIN, DENTON and HUBBARD (1953) took up an *in vitro* technique recording the contractions of the terminal ileum and the isolated gallbladder of the guinea pig in an intestinal bath, as elaborated by JUNG and GREENGARD (1933). They analyzed 7 samples of CCK against two standard samples using both the terminal ileum and the gallbladder. They found exactly the same strength expressed in CCK-units as found with the *in vivo* dog technique. The accuracy of the single determinations was also equally good.

In 1938 DOUBILET and IVY measured the intravesicular pressure in similar experiments using the isolated guinea pig gallbladder and ÅGREN (1939) registered the degree of contraction of the isolated gallbladder. Of his standard preparation 20 mg were added to a bath volume of 50 ml. He compared the effect with that on the gallbladder *in situ* in barbitalized cats with the duodenum cut open to permit direct inspection of the outflow of bile from the papilla of Vater and of the contracting gallbladder simultaneously.

A technique similar to that of ÅGREN (1939) and of GERSHBEIN *et al.* (1953) using isolated guinea pig gallbladder and intestine *in vitro* in an intestinal bath was applied by HULTMAN (1955 a). The contractions were recorded and read off in mm on a scale. Histamine and acetylcholine were simultaneously tested before and after adding Desentol Leo as an antihistaminic and atropine to the intestinal bath. HULTMAN paid particular attention to the sources of error such as changes in pH or in the salt concentration and the presence of ammonium salts. He like ÅGREN controlled his results in the anesthetized cat *in vivo* by observing the outflow of bile.

HULTMAN (1955 b) made the interesting observation that our highly purified CCK preparations exerted no substance P-activity on the intestine.

The relaxation of the sphincter Oddi, observed by SANDBLOM, VOEGTLIN and IVY (1935) to occur in man after injection of CCK, was also found by HULTMAN in cats in using the purified CCK samples.

The Ivy dog unit of cholecystokinin

In comparing the different techniques applied in assaying the biological activity of cholecystokinin, preference is to be given to that used in the laboratory of Dr. A. C. IVY whose experience now extends over a period of 30 years. The technique elaborated by GERSHBEIN *et al.* (1949) is easily reproducible and works with great accuracy. A full account of the details of the method used in assaying both secretin and cholecystokinin on cats and on dogs was recently given by IVY and JANECEK (1959).

The same preference must also be given to the dog unit of cholecystokinin suggested by IVY. It has a very suitable strength, because, as we have found, an optimal contraction of the gallbladder is usually obtained in man, when one Ivy dog unit of cholecystokinin per kg body weight is administered intravenously. This unit is defined as that amount of dry material (in the dose used) which when dissolved in normal saline solution and injected intravenously during 10 to 15 sec in a dog weighing about 15 kg results in a more or less immediate (1 to 3 min) rise in intragallbladder pressure of 1 cm of bile. The assay is made in the dog when the control pressure is from 7 to 10 cm of bile pressure.

Experimental

In assaying a sample of our purified cholecystokinin in 11 dogs, IVY and JANECEK (1959) found 45 μ to be the average dog unit, the strength thus being 22 Ivy dog units per mg.

We have applied the Ivy technique, slightly modified, for the comparison of the strength of two other samples of cholecystokinin (Cecekin Vitrum) with that of sample no. 15, which we use as standard. The results are shown in Table I.

Because of the great variations in weight of the dogs the amount of cholecystokinin injected was calculated per kg body weight. Furthermore two of the dogs were kept anesthetized and used continuously for 48 hours. They responded equally well at the end of the period as in the beginning.

As is seen from the table, there is a good proportionality between dose and response within the range of 2 to 6—7 cm increase in pressure. This corresponds to doses of 10 to 30 μg of sample no. 15 per kg body weight. 20 μg gave in the three dogs a response of about 6 cm, *i. e.* 300 μg in a 15 kg dog or one Ivy dog unit in 50 μg and 20 units per mg. The figure checks well with the strength of 22 units per mg as found by IVY and JANECEK in using 11 dogs.

The larger dose, 30 μg per kg body weight, approaches the upper limit for the proportionality line, an increase to 40 μg per kg persistently giving only a slightly higher or no further increase in pressure. If the same could

Table I. The assay of cholecystokinin in the anesthetized dog according to the Ivy technique

Dog No. 1 Weight 26 kg						August 15, 1958.								
Sample of cholecystokinin						No. 15								
μg cholecystokinin/kg						15	30							
Series of injections														
No. 1 Increase in pressure, cm..						5.0	10							
No. 2						5.0								
Dog No. 2 Weight 12 kg						August 21—23, 1958 (48 hours)								
Sample of cholecystokinin (Cecekin Vitrum)						No. 15				No. 26				
μg cholecystokinin/kg						10	20	30	40	10	20	30		
Series of injections														
No. 1 Increase in pressure, cm..						2.0	4.5	6.5	7.6		7.0	12.0		
No. 2						2.5	4.75	6.5	7.0	2.5	6.5	7.8		
No. 3						1.5	4.0	5.0		3.5	7.0			
No. 4						2.0	5.0	6.0			7.5	9.0		
No. 5						3.0	5.5	7.9			7.5	8.5		
No. 6							6.25	8.0			7.5	8.0		
No. 7							6.5	8.75			8.0	8.25		
No. 8							5.25	7.75			7.75	9.5		
						2.2	5.22	7.05	7.3	3.0	7.34	9.01		
Dog No. 3 Weight 26 kg						August 26—29, 1958 (48 hours)								
Sample of cholecystokinin (Cecekin Vitrum)	No. 15				No. 19				No. 26					
μg cholecystokinin/kg	10	20	30	40	10	20	30	40	10	20	30	40		
Series of injections														
No. 1 Increase in pressure, cm..	3.0	6.5	7.0	7.5	4.0	9.0	10.0		3.5	5.75				
No. 2	3.0	6.0	8.0		5.3	7.0			3.5	6.0	8.25			
No. 3	3.0	6.0	7.5		5.0	6.5	8.5		3.5	7.0	8.0	8.75		
No. 4	3.0	6.5	8.0		4.0	7.0								
No. 5	3.0	6.0	8.0		4.5	8.5								
No. 6					5.0	9.0	10.0							
No. 7					5.5	9.5	11.0	12.5						
No. 8						10.0	12.5	13.0						
	3.0	6.1	7.7	7.5	4.8	8.3	10.4	12.75	3.5	6.3	8.2	8.75		

be applied in man, a dose of 30 μg per kg body weight of cholecystokinin of standard strength (22 units per mg) should be the optimal clinical dose to be given intravenously in cholecystography. This corresponds to 2 to 2.5 mg of cholecystokinin or 44—55 Ivy dog units in a normal person.

The dose of our cholecystokinin samples empirically found to be sufficient is 2.5—3 mg (JORPES *et al.* 1958, JORPES and MUTT 1959). Even if a good response can be obtained in man with such a small dose as 0.5 mg or about

10 Ivy dog units or less (EDHOLM 1958), it may be safe to recommend the use of 1 Ivy dog unit per kg body weight or 60—80 Ivy dog units in total. A suitable strength of an ampoule of cholecystokinin is 75 Ivy dog units. This dose has up till now been given in more than 2,000 cholecystographies in man, and has been found to be sufficiently large. In cases with non-reacting gallbladder no better response was obtained with larger doses.

Side reactions

The occurrence of side reactions did in the past seriously hamper the use of secretin. Even our highly purified secretin preparations with about 3,300 Ivy dog units per mg can give a facial flush reaction. This is not due to the secretin itself, because a product giving no side reactions can be prepared. The same applies to cholecystokinin.

Even in the absence of histamine in the preparations such mild flush reactions are not uncommon. They do not, however, interfere with the clinical use of the product, and a slow speed of injection reduces their frequency. Nor do biliary concrements cause any pain after a cholecystokinin injection if not, as sometimes can be seen, a stone occludes the entrance of the cystic duct.

Pancreozymin and secretin in the cholecystokinin preparations

All our preparations of cholecystokinin so far obtained show a pancreozymin activity which seems to be closely correlated with the action on the gallbladder.

Secretin is not quantitatively removed from the cholecystokinin preparations.

IVY and JANECEK found about one-half to one unit of secretin to each unit of cholecystokinin in one of our samples. This would make 70 Ivy cat units of secretin, corresponding to not more than 300 or 350 Hammarsten units per ampoule of cholecystokinin, or about 1/3—1/5 of the amount necessary for an ordinary secretin test in normal persons. One ampoule of the commercial product, Cecekin Vitrum, contains about the same amount of secretin. IVY and JANECEK made the remarks "that the presence of secretin should be advantageous for the clinical use. It promotes a flow of pancreatic juice which resists biliary-pancreatic reflux and simulates physiological conditions".

Because of the extreme lability of secretin as witnessed by the last named authors it is, however, at present not feasible to warrant a definite amount of secretin in the cholecystokinin preparations unless an extra amount of it is added.

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A Rapid and Sensitive Electrode for Continuous Measurement of pCO_2 in Liquids and Tissue

By

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Abstract

Hertz, C. H. and B. Siesjö. A rapid and sensitive electrode for continuous measurement of pCO_2 in liquids and tissue. *Acta physiol. scand.* 1959. *47.* 115—123. — A pCO_2 electrode for measurements in tissues and liquids is described, based upon the principle first employed by Stow, Baer and Randall (1957) for pCO_2 measurements in liquids. Tissue measurements have been made possible by using a plane membrane pH glass electrode covered by a Teflon membrane. Together with a miniature calomel electrode it forms a pCO_2 electrode, which proved to be superior to earlier electrodes. Thus it is simpler in construction, more sensitive and about three times as fast as earlier electrodes.

Until recently there was no direct method for measuring carbon dioxide tension, and therefore continuous measurement of carbon dioxide tension (pCO_2) in liquids and tissues was impossible. In 1957, however, Stow, Baer and Randall described an electrode, which enabled direct measurement of carbon dioxide tension in liquids by an electrochemical method, based upon the fact that physically dissolved carbon dioxide changes the pH of an aqueous solution, proportional to log pCO_2 of the solution (Maxon and Johnson 1952, Toren and Heinrich 1957). The electrode utilized a gas permeable membrane allowing carbon dioxide to diffuse from the unknown sample into a thin

film of water on the surface of a conventional pH electrode. The measured pH was found to be altered in direct proportion to changes in log pCO_2.

Some modifications of the electrode have been reported. GERTZ and LOESCHKE (1958) used polyethylene instead of the original rubber membrane. An extensive study of the method was made by SEVERINGHAUS and BRADLEY (1958). They were able to increase the stability of the electrode and its speed of reaction by using a Teflon membrane and by adding sodium bicarbonate to the reference liquid. A similar electrode was briefly described recently by GRÄNGSJÖ and ULFENDAHL (1958).

The electrodes described so far, however, are difficult to construct and handle, rather slow, with a response rate of 2 to 15 min, and cannot be used for tissue recording because of the shape of the hemispherical standard pH electrode. In this paper will be described an electrode, which is small, easy to build and handle, suitable for continuous recording in liquids and tissue and about three times faster than those previously described. In addition, its high sensitivity should also make it suitable for clinical measurements of blood pCO_2.

Construction

In constructing the electrode special attention was paid to the following requirements: the electrode assembly should be as small as possible and its weight as low as possible; the exchange of any of its components should be affected easily and without removing any of its other components; the response time should be improved; the shape of the active area should also permit tissue measurement; finally, the electrode should be able to stand fairly strong mechanical shocks. To fulfil these requirements the electrode was constructed according to the following (Fig. 1 a).

The pCO_2 electrode consists of a plane membrane glass electrode G inserted in a plexiglass housing H and touching with its lower end a 0.006 mm Teflon membrane[1] M. Actually, because of the slight concavity of the pH glass membrane g a thin layer of about 0.1 mm is formed between the glass membrane g and the Teflon membrane M. A 5 mm bore hole in the side extension E in connection with the inside of the plexiglass housing H accepts the calomel reference electrode C. The plexiglass housing H is filled with a solution of 0.001 or 0.0001 N $NaHCO_3$ respectively up to a level of 2 cm above the lower end of the calomel electrode C. Since the space between the glass electrode G and the plexiglass housing H is only a few tenths of a millimeter, the amount of solution is very small and contributes very little to the weight of the assembly. On the other hand, this construction does not permit the use of the electrode in a horizontal, or upside down position. A cover with a hole for the thin coaxial cable L covers the upper end of the plexiglass housing H. The Teflon membrane M is kept in a tightly stretched position by a rubber ring R. To avoid leakage from outside into the reference liquid, vacuum grease is applied above the rubber ring R.

In order to get pH electrodes suitable for tissue measurements of pCO_2 a thin membrane of Corning 015 glass[2] is sealed onto the end of a heated soda glass tube

[1] Kindly supplied by A. B. Gerber and Hesslow, Stockholm.

[2] Kindly supplied by the Radiometer Co., Copenhagen.

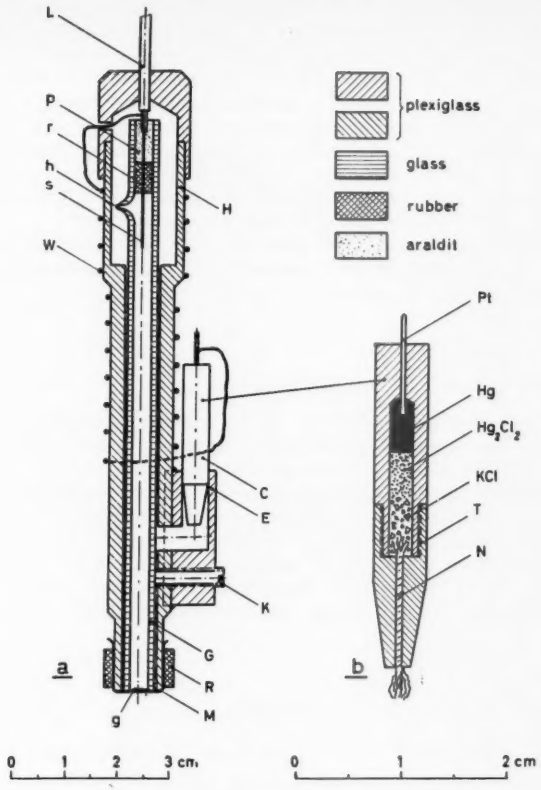

Fig. 1. The assembled pCO_2 electrode. H plexiglass housing, G glass electrode with plane active membrane g, C calomel reference electrode, M Teflon membrane. See text.

(6.5 mm O. D.) according to MacInnes and Dole (1930), care being taken to obtain glass membranes as plane as possible. As inner electrolyte 0.1 N HCl is used. The inner silver-silver chloride electrode sof the pH electrode is kept in place by a rubber stopper r, which is sealed in the soda glass tube by Araldit P with the lead to the reference electrode cemented into the plastic. A small branch h in the upper end of the glass tube facilitates the insertion of the rubber stopper r. Finally the glass electrode is kept in place in the housing H by a plexiglass screw K.

To obtain the pCO_2 electrode as stable as possible, a miniature calomel reference electrode was constructed and the instability experienced with silver-silver chloride electrodes (Strow *et al.* 1957) was thus avoided. As shown in Fig. 1 b the calomel electrode consists of two plexiglass rods (5 mm O. D.) which can be screwed together with the threads T. In the upper part of the assembly a 0.5 mm hole is drilled, through which is inserted a 0.5 mm platinum wire Pt. A 2.2 mm bore hole in the plexiglass is filled with

Hg and Hg_2Cl_2, upon which wet KCl crystals are tightly packed and kept in place by the two plexiglass pieces screwed together. To ensure contact between the potassium chloride in the electrode and the reference liquid of the pCO_2 electrode a nylon thread n is tightly inserted into a 0.5 mm bore hole in the lower end of the calomel electrode. Such reference electrodes can be used in any position and have proved to be very stable after being in contact with the reference electrolyte for some time to achieve constancy in diffusion potentials. The calomel electrode, prepared in this way, is fitted tightly into the hole in the extension E of the plexiglass housing H (Fig. 1) and its platinum wire is connected to the outer lead of the coaxial cable L via a spiral of fine wire W around the housing H, serving as an electrostatic screen.

The completed electrode assembly fulfils the mechanical requirements previously stated. With a 60 cm coaxial cable it weighs 27 g but its dimensions and weight can be decreased considerably if needed.

Calibration

The pCO_2 electrode was calibrated at 36° C in saline, equilibrated with different gas mixtures of known carbon dioxide concentrations. For calibrating the electrode, the equilibrated saline was transferred to another bath, since calibration in the original open water bath could give slight irregularities of the readings, probably due to electrical leakage caused by water vapour. The instrument used for measurement was a pH meter, Radiometer type PHM 22.

Around 36° C and with a carbon dioxide concentration of 6 per cent a temperature change by one degree in the calibration solution changed the reading about 2 mV. Thus it was sufficient to keep the temperature constant within $\pm$ 0.1° C.

Results

The linear relationship between pH and log pCO_2 found by others was confirmed with gas concentrations from about 2 to 8 per cent. Fig. 2 shows the calibration curves for four pH electrodes, denoted by the numbers 1 to 4. It can be seen that the curves 1, 2a and 3 are parallel, but do not coincide, due to the slightly varying asymmetry potentials of the pH electrodes. Coincidence could conveniently be reached by adjusting the measuring instrument to a fixed value for one of the gas mixtures used for calibration. The constancy of the slopes of the curves indicated that one single gas mixture was sufficient for calibration.

Full reproducibility could not be reached from day to day, if the pH electrodes were always left in the reference liquid. This is shown in Fig. 2 where curves 2a and 2b represent calibrations with the same pH electrode, 2a about two hours after assembling the pCO_2 electrode, and 2b after a week with the pH electrode left in place. The drift towards alkalinity was probably due to the fact that water dissolves an appreciable amount of alkali from the Corning 015 glass (MacInnes and Dole 1930). Filling the electrode with new reference liquid restored the calibration curve to its original value. In some cases a

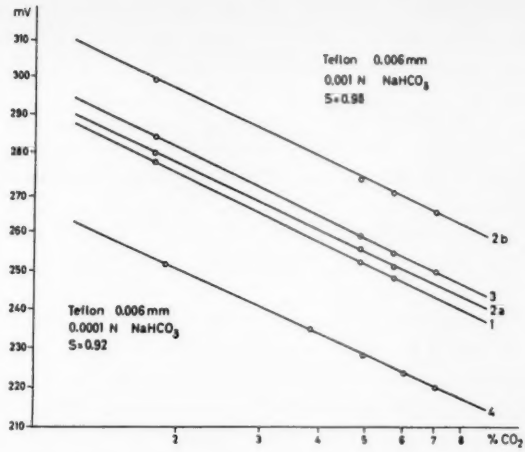

Fig. 2. Calibration curves for four different glass electrodes obtained in gas equilibrated saline. Curves 1 to 3 were obtained with a 0.001 N $NaHCO_3$ solution as reference electrolyte, curve 4 with a 0.0001 N solution. Note the insignificant decrease of sensitivity S with the more dilute solution.

small decrease of the asymmetry potential of the pH electrode might have added to the drift. Thus several daily calibrations were necessary. In many cases a faster shift, always to the alkaline side, was observed during the first two hours after assembling the electrode (*cf.* SEVERINGHAUS and BRADLEY 1958).

SEVERINGHAUS and BRADLEY (1958) defined the sensitivity S of a pCO_2 electrode as $\frac{\triangle \text{ pH}}{\triangle \log pCO_2}$, noticing a decreased sensitivity with decreasing bicarbonate concentrations in the reference liquid. Calculated according to this the sensitivity of the above described pCO_2 electrode with a 0.001 N $NaHCO_3$ solution in the reference liquid is practically unity, or the theoretical maximal value, which implies a higher sensitivity than that of previously described electrodes (Fig. 2, curves 1—3). This means that at 40 mm Hg, a 0.01 pH change is equivalent to a 0.94 mm change in pCO_2. The accuracy of the method is about $\pm$ 1 mV corresponding to $\pm$ 1.5 mm Hg at a carbon dioxide tension of 40 mm Hg.

With a 0.0001 N $NaHCO_3$ solution in the reference electrolyte the sensitivity in the carbon dioxide range used decreased very slightly to 0.92 which still is close to theoretical maximum and considerably higher than earlier reported sensitivities with this bicarbonate concentration. However,

at higher carbon dioxide tensions a slight deviation from linearity can be observed. This can be determined theoretically from the sensitivity equation for $NaHCO_3$ concentrations lower than 0.01. We find

$$S = \frac{\triangle\ pH}{\triangle\ \log\ pCO_2} = -\frac{[H^+] + [Na^+]}{2\ [H^+] + [Na^+]}$$

which is derived from the equation (SEVERINGHAUS and BRADLEY 1958)

$$\alpha\ pCO_2 = [H_2CO_3] = \frac{[H^+]^2 + [H^+][Na^+] - K_w}{K_1\ (1 + \frac{2\ K_2}{[H^+]})}$$

Here K_w is the dissociation constant of water, K_1 and K_2 the first and second dissociation constants of carbonic acid and α a constant denoting the proportionality between carbon dioxide tension and carbonic acid concentration. From this is found, that with a 0.0001 N $NaHCO_3$ solution the theoretical sensitivity varies somewhat with carbon dioxide concentration, being 0.98 at a carbon dioxide concentration of 1.8 per cent, 0.96 at 4.8 per cent, 0.95 at 7 per cent and 0.8 at 100 per cent. This slight deviation from linearity is, however, of no importance in biological work, since it is only observable at carbon dioxide concentrations higher than about 15 per cent. The accuracy of the measurement was not decreased by using the lower sodium bicarbonate concentration.

The *response time* of the pCO_2 electrode, estimated as the time needed for full equilibrium for a fourfold increase in pCO_2, depends on bicarbonate concentration in the reference electrolyte, as reported by SEVERINGHAUS and BRADLEY (1958). These authors used a 0.01 N $NaHCO_3$ solution because of instability and greatly reduced sensitivity at lower concentrations. The response time of the electrode, described here, was tested with three bicarbonate concentrations, 0.001, 0.0001 and the above named 0.01 N (Fig. 3). As can be seen, substituting the 0.01 with a 0.001 N solution made the electrode about 50 per cent faster. The 0.0001 N solution increased the response rate almost four times. In this last case 90 per cent equilibrium was reached in 25—30 sec with an instantaneous reaction. This was found for an increase in pCO_2, for a decrease the electrode is somewhat slower.

Thus the described electrode was faster than any of the previously described electrodes. This gain was achieved with an insignificant reduction of sensitivity (Fig. 2, curve 4). There was however, with the most dilute bicarbonate solution an initially fast and, later on, slow drift towards alkalinity. (See Discussion.)

On account of the drift we used the 0.001 N $NaHCO_3$ solution for discontinuous carbon dioxide measurements. The recording of fast changes of pCO_2 in tissues and liquids, however, necessitated the inclusion of the 0.0001 N $NaHCO_3$ solution. In this case even distilled water could be used as reference

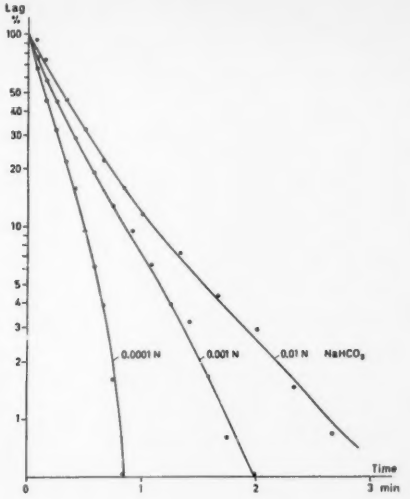

Fig. 3. Response time of the electrode in saline with different sodium bicarbonate concentrations in the reference electrolyte obtained for a four-fold increase in CO_2 tension.

solution, and then response time was further reduced to about 30 sec for final equilibrium after a fourfold increase in pCO_2. The gain in speed with distilled water was, however, counteracted by a decreased sensitivity.

We have confirmed the findings of others that the readings are identical in gases and different liquids including blood. The electrode can thus be used to measure the CO_2 tension of blood, liquids and probably also tissues and can be calibrated in gas streams with due precautions.

Discussion

Previous pCO_2 electrodes have been designed for discontinuous blood measurements. Their application to continuous measurements and tissue measurements has been impossible because of the slowness of the electrodes, the shape of the pH electrodes and the complex constructions of the assemblies.

The present electrode has been designed to permit also tissue measurements by including a plane membrane glass electrode in the assembly, and by increasing the response rate. Tissue measurements would be of special interest in the study of organs which mainly metabolise glucose, *e. g.* the brain, where carbon dioxide is one of the principle end products of metabolism and further supposed to be linked to the local regulation of the circulation (Kety 1956, Ingvar 1958). An account of continuous measurements of the carbon dioxide tension of the cerebral cortex will appear shortly (Ingvar, Siesjö and Hertz 1959).

The high sensitivity of the electrode is probably dependent upon the shape

of the pH electrode. Between the slightly concave pH glass membrane and the Teflon membrane a layer of about 0.1 mm is formed, holding a constant electrolyte volume. This has made unnecessary the cellophane layer, which has been found to decrease sensitivity (SEVERINGHAUS and BRADLEY 1958).

Among other things the response time of the pCO_2 electrode depends on sodium bicarbonate concentration, membrane material and membrane thickness. This was noticed by SEVERINGHAUS and BRADLEY (1958) who, however, had to use a 0.01 N $NaHCO_3$ solution as an appreciable loss of stability and sensitivity occurred at lower concentrations. This was probably due to the cellophane necessary to hold a constant layer of reference liquid at the surface of the pH electrode. Further, in very dilute electrolytes silver-silver chloride reference electrodes give rise to additional instability (STOW, BAER and RANDALL 1957). By adding a stable calomel reference electrode and by removing the cellophane layer it was possible to use a 0.0001 N $NaHCO_3$ solution. The response time of the electrode was thereby decreased about three times with only about 5 per cent loss of sensitivity. The drift observed with this bicarbonate solution is in agreement with the experienced difficulty of measuring accurately the pH of unbuffered, or slightly buffered solutions with glass electrodes (MACINNES and DOLE 1930, ELLIS and KIEHL 1935, KRATZ 1950). Since different types of pH glasses have been found to give off varying amounts of alkali (KRATZ 1950) this drift could probably be overcome by choosing another type of pH glass than Corning 015 glass, which has a specially high solubility.

The main mechanical advantage of a pCO_2 electrode, constructed as described above, is that the pH electrode can be exchanged very easily. The pH electrode is just lowered into the plexiglass housing, pressed against the Teflon membrane and fastened by the plexiglass screw. The small pressure applied to the Teflon membrane permits the use of thinner membranes than those previously used, which improves response time.

It is a pleasure to acknowledge the support and interest of Dr. D. H. INGVAR, who originally suggested this work. We are indebted to Dr. ing. S. SCHRÖDER of the Radiometer Co. in Copenhagen for his active interest. Miss B. SÖRENSSON gave valuable technical assistance. The work was supported by grants from the Swedish Medical and Swedish Natural Science Research Councils.

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Observations on the Distribution and Action of Substance P in Marine Animals[1]

By

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Abstract

DAHLSTEDT, E., U. S. v. EULER, F. LISHAJKO and E. ÖSTLUND. Observations on the distribution and action of Substance P in marine animals. *Acta physiol. scand.* 1959. *47.* 124—130. — Various parts of the CNS of the dogfish and the ray were found to contain large amounts of Substance P (SP), up to 50 units/g fresh organ. It was also found in extracts of intestine of these species, and in minute amounts in the hagfish (*Myxine*). No SP activity was found in total extracts of *Mytilus* and *Actiniaria*, but small amounts were found in *Ciona*. The strongly stimulating action of SP on teleost intestine was confirmed with superfusion technique.

In a previous communication it has been reported that Substance P (SP) is present in extracts of intestine and brain in various species of fish (EULER and ÖSTLUND 1956). High amounts (250 units/g) were found in the brain of *Rana esculenta* by CORREALE (1956). Moreover, it has been observed that SP effectively stimulated the motility of the isolated intestine in several fish species (EULER and ÖSTLUND 1957).

The present paper gives some further data on the occurrence of SP in various marine animals and particularly in different parts of the central nervous system in Elasmobranchs. By comparing the amounts of SP in various repre-

[1] A preliminary report has appeared in II. Internat. Sympos. Neurosekretion, Lund (1957).

Table I. Substance P in various parts of the central nervous system of Squalus acanthias and Raja batis

Animal	Preparation	Content of Substance P in units per g
Squalus acanthias	Telencephalon	50
» »	Mid-brain and cerebellum	21
» »	Medulla oblongata	45
» »	Spinal cord	18
Raja batis	Whole brain	4.0, 9.6, 7.0
» »	Telencephalon	5.1
» »	Mid-brain and cerebellum	8.5
» »	Medulla oblongata	39
» »	Spinal cord, dorsal part	25
» »	» » ventral »	15
» »	Spinal cord	19, 26, 28

sentatives of marine animals it was hoped to gain some information of the appearance of this substance in the animal series. In addition some observations are reported on the effect of SP on the motility of the isolated intestine of some teleosts.

Material and methods

The material was obtained fresh from the sea and immediately prepared at the laboratory. The whole bodies of *Actiniaria* and *Ciona* were used after removal of excess water. Of *Mytilus* the soft tissues only were prepared.

The spinal cord and the brain were dissected out and prepared from freshly killed specimens of dogfish (*Squalus acanthias*) and ray (*Raja batis*). Extracts were also prepared of the dorsal and ventral parts of the spinal cord and of the medulla oblongata of the ray. The olfactory brain, the medulla oblongata and the intermediate part of the brain in the dogfish were also prepared. In addition, the brain and the intestine of the hagfish, and the intestine of the pike, the dogfish and *Nephrops norwegicus* were extracted and analyzed for their content of SP.

All material was cut in rice-grain size pieces with scissors and boiled for 10 min in 5 volumes of water acidified with sulphuric acid to pH 4, at which reaction SP is stable even during prolonged boiling (Gaddum and Schild 1934). After cooling and filtering, the extract was saturated with ammonium sulphate and left overnight in the cold-room. The precipitate was filtered off and washed repeatedly with 2/3 saturated ammonium sulphate. The dried precipitate was dissolved in 4 volumes of water under slight warming, and methanol added in small portions to 70 per cent v/v with stirring. After settling in the cold overnight the voluminous inactive precipitate was centrifuged off, washed with 70 per cent methanol and again centrifuged. The clear supernatants were passed through a column containing 10 g of aluminium oxide (B. D. H.) and eluted with 20 ml portions of falling concentrations of methanol from 60 per cent to 20 per cent followed by repeated washings with water according to Pernow (1953).

After removing the methanol by evaporation in vacuo the eluates were used for bioassay on the isolated guinea-pig ileum. A 3 ml bath was used with Tyrode's solution

Table II. Elution of Substance P activity from aluminium oxide column after adsorption of extract of 11 g spinal cord of Raja batis in 70 % methanol

Methanol conc.	Volume ml	Activity units
60 %	20	—
50 %	»	—
40 %	»	4.5
20 %	»	24
H_2O (V-1)	»	240
H_2O (V-2)	40	30
H_2O (V-3)	»	12
Total		311

at 38° C, bubbled through with oxygen. The bath fluid contained atropine sulphate and an antihistaminic (Lergigan®) in concentrations of 0.4 mg per l (PERNOW 1953). A number of extracts were also tested on the blood pressure of the atropinized rabbit, and on the isolated jejunum of the rabbit.

In order to test the specificity of the effects obtained, the extracts were incubated at 38° C with trypsin (Trypure Novo) for 10 min to 1 hour which causes inactivation of SP (PERNOW 1955). The trypsin concentration was 0.2 mg per ml in the incubate. The extracts were tested against a SP standard containing 10 units per mg.

The effects of purified preparations of SP containing 100 units per mg were tested on isolated pieces of small intestine from *Pleurinectes platessa* and *Labrus berggylta* suspended in 50 % deep sea water at 20°—22° C. The effects of SP on the intestine were tested either by addition to the bath fluid or by letting a small volume of the active solution (0.005—0.01 ml) trickle over the surface of the intestine after emptying the bath, similarly to the superfusion technique of GADDUM (1953).

Results

Substance P in the central nervous system of Elasmobranchs

The results of the analysis of the SP content in the spinal cord and in different parts of the brain of *Squalus acanthias* and *Raja batis* are summarized in Table I.

The amount of SP was also determined in the brain of the hagfish (*Myxine glutinosa*). A total of 3.0 g brain from 173 specimens contained 3.3 units per g.

The results of adsorption and elution of an extract of 11 g spinal cord from 6 rays weighing 40 kg is shown in Table II, and illustrated in Fig. 1.

The occurrence of Substance P in various marine animals and in organs other than the central nervous system

The following animals and organs were extracted and tested for their content of SP.

Whole animals: *Mytilus edulis*, 0.34 kg, *Actiniaria* 1 kg, *Ciona intestinalis* 1.3 kg.

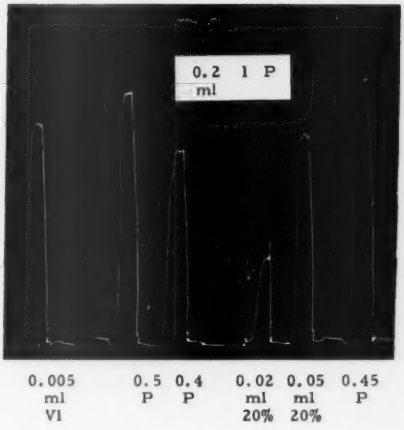

Fig. 1. Isolated guinea pig ileum, 3 ml bath, Tyrode, 38° C. Atropine and Lergigan® 0.4 mg/l. V-1, first aqueous elution fraction (3 ml). 20 %, weakest methanol elution fraction (3 ml). Extract of spinal cord of *Raja batis* adsorbed on alumina. P, units of Substance P Standard. Inserted: Effect on rabbit's blood pressure of extract and standard in doses equiactive on guinea-pig ileum.

Intestine: *Myxine glutinosa* 0.25 kg, *Nephrops norwegicus* from 8 kg animals, *Esox lucius* 0.2 kg, *Raja batis* 90 g.

Ventricle: *Raja batis* 0.1 kg.

Table III shows the results obtained by bioassay using purified extracts of the whole animals and the intestine or ventricle of the animals listed above.

The results shown in Table III confirm previous findings of SP in extracts of intestine of fish (EULER and ÖSTLUND 1956). While the amounts of SP is from 0.72—3.0 units per g in *Esox* and in the Elasmobranchs it is only 0.04 units/g in *Myxine*. No activity was found in the intestine of *Nephrops*. The ventricle of *Raja batis* contained some SP, but less than the intestine.

In extracts of *Mytilus*, *Actiniaria* and *Ciona*, Substance P-like effects were only observed with certainty in the last mentioned species.

Effect of Substance P on the motility of the isolated intestine of teleosts

The previous observations that SP stimulates the motility of the intestine of teleosts were confirmed. The threshold dose when SP was added to the bath fluid was about 0.25 units per ml in tests on the small intestine of *Pleuronectes* and *Labrus*.

By applying the extract to the surface of the intestine suspended in air the amounts of SP necessary to produce contraction were greatly reduced; doses as small as 0.1 unit being sufficient to cause a marked effect. As might be expected the effect was dependent on the concentration of SP in the solution applied, when the volume was kept constant (Fig. 1). Using the same concentration the action varied with the amount, however, allowing semi-quantitative estimations.

The type of action caused by SP was generally one of sustained activity. The onset of the contraction was relatively rapid although less brisk than for *e. g.* serotonin or acetylcholine.

Table III. Substance P content of marine animals

Animal	Part of animal	Substance P content units per g
Mytilus edulis	Whole animal	< 0.01
Actiniaria	» »	< 0.01
Ciona intestinalis	» »	0.02
Nephrops norwegicus ..	Intestine	< 0.1
Raja batis	»	3.0
» »	Ventricle	1.3
Myxine glutinosa	Intestine	0.04
Esox lucius	»	0.72
Squalus acanthias	»	2.0

Discussion

The identity of the active substance found in comparatively large amounts in the central nervous system of Elasmobranchs with Substance P in mammalian intestine and brain is supported by the parallelism in the following respects

Method of preparation,
» » purification,
Breakdown by trypsin,
Atropine and antihistamine resistance,
Stimulating effect on the rabbit and guinea pig intestine,
Lowering of rabbit's blood pressure.

Although the extracts of brain of *Myxine* were inactivated by trypsin and showed the biological properties of SP, the eluate fraction in which the main activity appeared differed from that for other organs. The maximum was found in the 40 % methanol fraction while in all other extracts it appeared in the first water fraction. It can therefore not be excluded that the "Substance P" in *Myxine* has a different composition from that in the Elasmobranchs.

SP is not evenly distributed in the central nervous system of the Elasmobranchs studied. In the spinal cord the dorsal part contained more than the ventral part, suggesting different concentrations in the dorsal and ventral roots. It may be recalled that PERNOW (1953) and LEMBECK (1953) found considerably higher amounts of SP in the dorsal than in the ventral roots of mammals, from which the tentative conclusion was drawn that it might play a role for the transmission process in sensory neurons.

Marked differences also occur in the distribution of SP in different parts of the brain. Thus telencephalon and medulla oblongata in *Squalus* contained

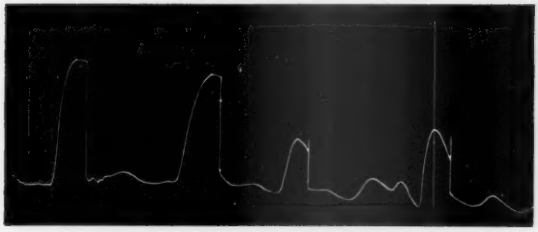

Fig. 2. Isolated small intestine of *Pleuronectes*, mounted in air, intermittently washed in 1/2 deep sea water. Temp. 22° C. Topical administration of Substance P (volume and units) and acetylcholine as indicated.

about twice as much SP as the midbrain with cerebellum and the spinal cord. Low figures in the cerebellum of mammals were found by PERNOW (1953), KOPERA and LAZARINI (1953) and by ZETLER and SCHLOSSER (1954). Although central effects have been reported by EULER and PERNOW (1956), ZETLER (1956 a, b) and by STERN and DOBRIČ (1957), further experiments are needed in order to relate changes in concentration in various parts of the central nervous system to specific functions.

The superfusion experiments confirm the usefulness of this technique. Topical application of a solution of SP on the surface of the exposed mammalian intestine in situ has previously been shown to induce a marked increase both in peristaltic movements and in the appearance of local contraction (GERNANDT 1942).

The present data have demonstrated that SP occurs in intestine and central nervous system of teleosts and Elasmobranchs. In the more primitive hagfish SP was also present, but in small amount. Particularly interesting was the finding that extracts of *Ciona* exerted what appeared to be typical SP effects, in view of the fact that this animal belongs to the chordates, while no such action could be detected in extracts of *Actiniaria* and *Mytilus*. It may be concluded that Substance P is an active substance which appears at a certain stage in the developmental chain of marine animals.

Summary

1. The spinal cord and the brain of the dogfish (*Squalus acanthias*) and the ray *Raja batis* contain high amounts of Substance P (SP), up to 50 units per g.

2. The dorsal part of the spinal cord of *Raja batis* contains about twice as much SP as the ventral part.

3. The brain of the *Myxine glutinosa* contains the equivalent of 3 units of a SP-like substance.

4. The intestine of *Raja batis*, *Esox lucius* and *Squalus acanthias* contains 0.72—3 units SP per g. *Myxine* contained less than 0.1 unit per g.

5. Small but definite amounts of SP were found in extracts of *Ciona intestinalis* but not in *Mytilus* and *Actiniaria* or intestine of *Nephrops*.

6. The stimulating action of SP on the isolated intestine of some teleosts was confirmed.

We are greatly indebted to Dr. G. Gustafson and the Staff of the Kristineberg Zoological Station for their kind co-operation in the collection of material.

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The Effect of Functionally Induced Changes of Wall/Lumen Ratio on the Vasoconstrictor Response to Standard Amounts of Vasoactive Agents

By

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Abstract

FOLKOW, B. and B. ÖBERG. The effect of functionally induced changes of wall/lumen ratio on the vasoconstrictor response to standard amounts of vasoactive agents. *Acta physiol. scand.* 1959. *47.* 131—135. — It has recently been suggested that the functionally induced increase in wall/lumen ratio, obtained whenever vascular tone is increased, might explain the vascular 'hyperresponsiveness' seen in hypertensive disease. The present experiments indicate, however, that this is not the case, presumably because the smooth muscle shortening to a given excitatory stimulus gets smaller the higher the initial tone is. To create a structurally based vascular 'hyperresponsiveness' a true increase of the wall mass of the resistance vessels is a necessary prerequisite.

To judge from histological examination of arteries and arterioles in hypertensive disease (see PICKERING 1955), a gradual increase of the vascular wall mass takes place, causing an increased wall/lumen ratio. The haemodynamic consequences, which have recently aroused some interest (for lit. see FOLKOW, GRIMBY and THULESIUS 1958), are best judged if comparisons of vessels with normal and thickened walls start from a common, well defined reference point. The maximally dilated state, where the often unpredictable variable of smooth muscle tone is eliminated, forms such a 'baseline' for comparison as under such circumstances only structural-physical characteristics are of relevance. It is then found that an increased wall mass can affect the haemodynamic characteristics of a vessel in two different ways (FOLKOW 1956):

1) The growth of the vascular walls in hypertension might expand also in an inward direction. The resistance to flow will then be higher than in normotension even when vascular tone is completely abolished, and will be so at all levels of vascular smooth muscle activity. TOBIAN and BINION (1952) suggested that a water-logging of the arteriolar walls might lead to such consequences.

2) Even if the increase of the vascular wall mass has not interfered with the lumina in the maximally dilated state, it will nevertheless exert most important haemodynamic effects. The increased amount of tissue situated inside the 'line of force' for vascular smooth muscle contraction will, for any given muscle shortening, cause intensified reductions of the lumen as compared with normal thin-walled vessels. Experimental results (FOLKOW *et. al.* 1958) suggest that in so-called essential hypertension both, or at least the second, of the above mentioned mechanisms are engaged to an extent dependent on the increase of vascular wall mass. As most authors agree that the walls of the arterioles in hypertensive disease really get thicker, this means that purely physical factors may contribute significantly to the increased resistance to flow.

The 'potentiating' effect of an increased wall/lumen ratio according to mechanism 2 has been further discussed by CONWAY (1958) and REDLEAF and TOBIAN (1958). The latter authors even suggested that the increase of wall/lumen ratio, obtained whenever vascular smooth muscle cells contract, might similarly 'potentiate' the vascular reactions to a given excitatory influence. If this were the case, any increase of vascular tone would automatically imply an intensification of vascular responses without necessitating any increase of total wall mass or any true sensitization of the muscle cells. This is an interesting possibility but there is so far no direct experimental support of such a view, and experiences from other studies in this laboratory make it unlikely that the vascular bed should behave in the suggested way. Even if the luminal reductions, caused by a given shortening of the vascular smooth muscles, certainly get stronger the more pronounced the initial constriction level is, it should be remembered that there is normally at least one contrary directed variable engaged: The higher the initial activity level of the contractile elements is, the smaller the additional shortening, induced by a standard quantum of excitatory influence, tends to become. — For the analysis of the changed characteristics of the vascular bed in acute and chronic hypertension it is important to know what will be the net effect of these two opposing factors in flow resistance.

Method

The experiments were performed on 12 cats under chloralose-urethane anesthesia (40 mg + 100 mg/kg body weight). A tracheal cannula was inserted and the carotid arteries and the vagal nerves were dissected free. Blood pressure was recorded from the left femoral artery by means of a mercury manometer. The blood flow from the

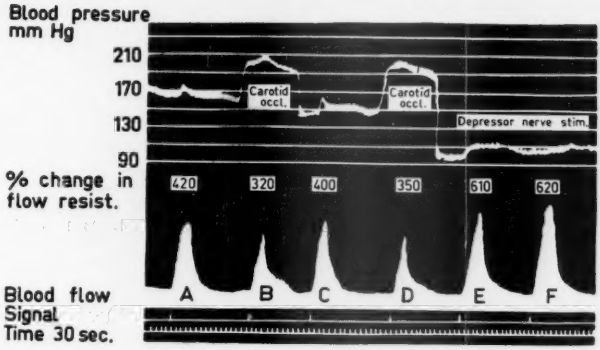

Fig. 1. Cat 3.2 kg. Chloralose-urethane. Records of blood pressure and blood flow in the right hind limb. Changes in flow resistance when intra-arterial injections of 2.5 μg are given at different levels of vascular tone.

right hind limb was measured by a closed optical drop recorder-ordinate writer unit, inserted between the two ends of the cannulated femoral vein. A fine polyethylene catheter was introduced into the right femoral artery through a small sidebranch on the thigh, thus allowing close arterial injections of vasoactive agents. — By the simultaneous recording of blood pressure and blood flow through the right hind limb its vascular resistance could be estimated continuously.

Different levels of vascular tone were induced, generally by reflex changes of vasoconstrictor fibre activity. From these different levels of flow resistance and perfusion pressure the effects of close arterial injections of standard amounts vasoactive agents on the blood flow through the hind limb were measured. Firstly a series of injections was performed with the cat in the 'normal' state, *i. e.* where the vascular bed was exposed to the 'resting' tonic vasoconstrictor fibre discharge and perfused at a normal pressure head. Secondly a series of injections was undertaken when the vascular bed was exposed to a raised sympathetic vasoconstrictor fibre activity with a proportionally raised blood pressure, produced by a bilateral occlusion of the carotid arteries. Thirdly, injections were made while the vasoconstrictor fibre activity was to some extent inhibited and therefore vascular tone and blood pressure were proportionally lowered, induced by a continuous afferent stimulation of the vagal depressor fibres. — In other experiments the initial level of flow resistance and perfusion pressure was increased by a constant intravenous infusion of noradrenaline or angiotensin.

The effects of standard amounts of vasoconstrictor substances could thus be studied on a vascular bed where the state of initial smooth muscle tone, and wall/lumen ratio, was varied in different ways within wide limits. Repeated injections were always performed at the different levels of tone to avoid chance variations in the vascular responses to the constrictor substance.

Results

The results from a typical experiment are shown in Fig. 1. A and C in the figure show the effects of intra-arterial injections of 2.5 μg of noradrenaline while the vessels were exposed to the 'resting' vasoconstrictor fibre activity.

Table I

Initial vascular tone:		Effect of 2.5 μg noradrenaline:	
		% increase of flow resistance	% decrease of internal radius
'Low'	E, figure I	610	39
	F, figure I	620	39
'Normal'	A, figure I	420	34
	C, figure I	420	33
'High'	B, figure I	320	30
	D, figure I	350	31

B and D illustrate the reactions to an intra-arterial injection of the same amount of noradrenaline when vascular tone was increased; E and F, finally, show the effect of noradrenaline when sympathetic tone was partly inhibited. The regional flow resistance in B and D is about twice as high as in E and F. — The percentage shifts in regional flow resistance between the initial state and the peak constriction were calculated from the flow and pressure values and the corresponding relative shifts in internal radius were also deduced (see Table I). It is obvious from this table that the higher the initial tone of the vascular smooth muscles is, — and consequently also the wall/lumen ratio —, the smaller in fact is the constrictor response induced by a standard amount of a vasoconstrictor agent. The results were in this respect quite consistent in all experiments, through they of course varied quantitatively to some extent from one animal to the other. The same effects were seen if the initial vascular tone, — and wall/lumen ratio —, had been raised by a constant intravenous infusion of noradrenaline or angiotensin, and the relationships were not changed if angiotensin was used as the test substance instead of noradrenaline. — It should be stressed that the range of the induced acute shifts in both the blood pressure and vascular tone levels, at which the standard amounts of constrictor substances were tested, are comparable to what is seen in normotension in comparison with moderate and strong chronic hypertension in man.

Discussion

For purely physical reasons it seems unavoidable that any process causing an increased wall/lumen ratio in a blood vessel will so affect its haemodynamic characteristics, that a given vascular smooth muscle shortening induces a relatively more pronounced lumen decrease (FOLKOW 1956, FOLKOW *et al.*

1958). It will do so once the vascular wall mass inside the line of force for smooth muscle contraction has increased, whether it is due to smooth muscle hypertrophy, intimal thickening, atheromatosis, water-logging or other processes, and this physical-structural change implies an apparent vascular hyperresponsiveness. Also the purely functionally induced increase in wall/lumen ratio, which is obtained whenever vascular tone is increased will necessarily have the consequence that a given additional smooth muscle shortening reduces the lumen relatively more than if the vessel is initially relaxed. The present experiments indicate, however, that this does not lead to any apparent hyperresponsiveness to a given excitatory influence, as REDLEAF and TOBIAN (1958) have suggested. On the contrary, the vascular response to a standard amount of constrictor agent actually decreases under such circumstances. This is in all probability due to the fact that the actual shortening of a muslce cell gets smaller the more contracted the muscle cell is to start with, and that this factor overcomes the intensifying influence of the simultaneously increased wall/lumen ratio. The net balance between these two opposing factors will thus lead to diminished constrictor responses at higher tone levels, at least as long as the vascular tone is kept within the limits actually met with in normo- and hypertension in man. The hypothesis of REDLEAF and TOBIAN, which was presented to explain the 'hyperresponsiveness' of the vessels of hypertensive subjects without necessitating any real increase of wall mass or any true sensitization of the muscle elements, is therefore disproved. Where, on the other hand, the vascular wall mass really has increased, whatever its cause, a given excitatory influence will cause intensified lumen reductions, provided the contractile elements are not less sensitive than those of normal vessels.

The authors are indebted to professor W. S. PEART, St. Mary's Hospital, London, for a generous supply of angiotensin.

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Studies on Slow Potentials in the Rabbit's Olfactory Bulb and Nasal Mucosa

By

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Received 31 March 1959

Abstract

Ottoson, D. Studies on slow potentials in the rabbit's olfactory bulb and nasal mucosa. *Acta physiol. scand.* 1959. *47.* 136—148. — When the nasal mucosa in the rabbit is stimulated with odorized air the olfactory bulb develops a slow potential with superimposed regular waves. The slow potential may be obtained in isolation after the intrinsic activity of the bulb and the induced waves have been blocked. If the bulb is removed and recording made from the cut end of an olfactory nerve bundle a slow positive potential is recorded that has the same time course and shape as the bulb response. The potential recorded from the nerve arises in the olfactory epithelium and is electrotonically conducted along the nerve fibres. The response of the receptor layer can also be obtained in direct recordings with the electrode pushed into the mucosa through one of the canals in the *ethmoid* bone. The receptor potential in the rabbit exhibits the same characteristics as the corresponding response in the frog.

It has been demonstrated by Adrian (1942, 1950, 1956) that large sinusoidal oscillations are produced in the olfactory bulb by stimulation of the nasal mucosa with odorized air. These potentials are most prominent in the grey matter and have therefore been ascribed to the activity in the dendritic feltwork. From the deeper layers of the bulb where secondary neurons are located Adrian (1951, 1953) recorded impulses in groups with the frequency of the waves. In addition to the waves and the spike potentials olfactory stimulation

also gives rise to a slow potential change recordable from the surface of the bulb (Ottoson 1954).

The present investigation was undertaken in order to analyse the sequence of electrical events leading to the production of the bulb response. Recordings have been made from the surface of the bulb and from the receptor layer in the nasal mucosa. The experiments to be reported show that there is a close correlation between the sustained potential in the receptor organ and the slow bulb response. Data are also presented on the functional properties of the olfactory end organs as revealed by the responses induced by various modes of stimulation.

Methods

The experiments have been carried out on rabbits narcotized with urethane or pentobarbital sodium. The olfactory bulbs were exposed and covered with mineral oil and recordings made with agar-AgCl-Ag-electrodes. The reference electrode was usually placed at the edge of the scalp. The potentials were amplified with a direct coupled amplifier (Haapanen 1953) and the responses photographed on moving film.

Olfactory stimulation was obtained either by making the rabbit inspire odorized air through the nostrils or by blowing the air into the nose. As a rule the latter method was used because the stimulation was then independent of the rate and depth of breathing. A respiration pump was employed for short single or repeated stimulation, whereas stimulation of longer duration was obtained by passing a constant stream of air through the nose. The air escaped through a tracheal cannula that was passed towards the back of the nose. Respiration, either normal or artificial, took place through another cannula. By connecting the two cannulae the rabbit was made to breathe through the nose.

The air used for stimulation was purified with activated carbon and then passed over a solution of the stimulating substance. Butanol, oil of cloves or methyl benzoate was mostly used. The air current could be shifted with stopcocks from purified to odorized air and passed into either or both nostrils. In most experiments stimulation was marked on the lower beam of the oscilloscope by recording the pressure change in the nose cannulae with a capacitance meter.

The anatomy of the olfactory organ and bulb in the rabbit

The olfactory epithelium in the rabbit is distributed over a comparatively large area of the mucous membrane and lines a series of complicated folds in the lateral and upper part of the nasal cavity. The receptor cells are bipolar neurons (Fig. 1) with extremely slender axons that pass through the basement membrane and form the olfactory nerve fasciculi in the mucosa. These bundles run through small holes in the *ethmoid* bone and spread out over the surface of the bulb. From here they pass deeper into the bulb and split up into numerous ramifications. The afferent terminals form together with the dendrites of the mitral cells well circumscribed spherical bodies, the glomeruli. These are considered as the most typical and distinctive structure of the bulb. The layer of glomeruli is succeeded by the plexiform layer with the tufted cells that together with the mitral cells in the following layer send axons centrally in the olfactory tract. The bulb also receives centrifugal fibres, probably from basal rhinencephalic areas.

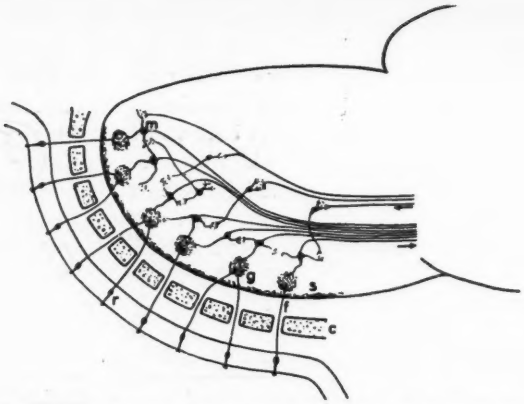

Fig. 1. Schematic diagram showing the general organization of primary and secondary olfactory neurons in the rabbit. r, bipolar receptor cell; f, fila olfactoria; g, glomerulus; m, mitral cell; s, superficial layer of afferent fibres; c, cribriform plate.

Results

A. General features of the bulb response

As described in a previous paper (OTTOSON 1954) stimulation of the nasal mucosa with odorized air sets up a slow potential in the bulb. This potential is positive in recordings from the dorsal and lateral surface of the bulb, while the anterior part produces negative potentials. Because most of the recordings in the present study were made from the dorsal surface only the positive potential will be dealt with in the following.

The olfactory bulb potential evoked by natural stimulation varies greatly with the depth of anaesthesia and from one preparation to another. The records in Fig. 2 illustrate typical responses obtained from three different preparations and with different odorous stimuli. Record A shows the most common type of response. In this case butanol was used as stimulus and the stimulation had a duration of about 1.2 sec. The odorized air blown into the nose gives rise to a sustained monophasic potential with superimposed waves. Usually the waves are highest on the top of the slow potential and then decline. Sometimes they wax and wane rhythmically as illustrated in B, while in other cases the amplitudes alternate regularly (C).

The olfactory bulb shows no conspicuous signs of fatigue under repeated stimulation. Responses of surprisingly constant features are thus produced (Fig. 2 D) when the nose is stimulated with a puff of air at each stroke of the pump. This is of interest in view of ADRIAN's (1950) observation that the secondary olfactory neurons in the bulb discharge with distinct bursts at each inspiration over periods of more than half an hour.

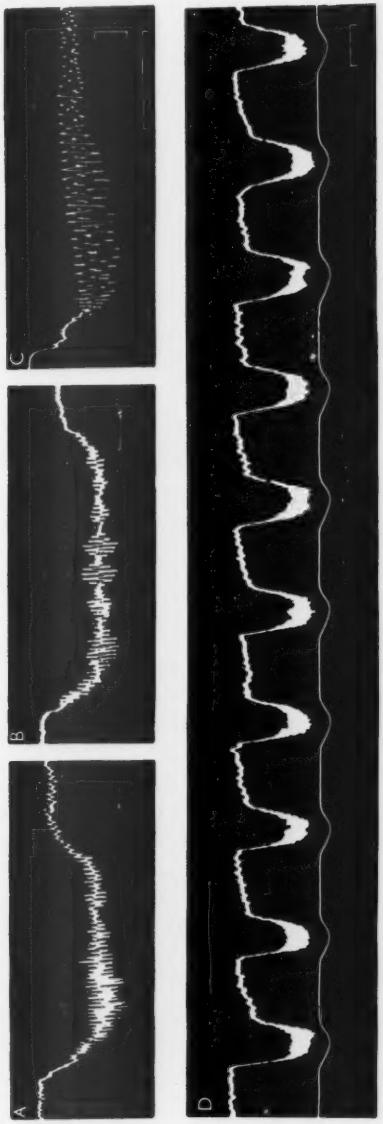

Fig. 2. Bulb responses evoked by blowing odorized air into the nose. Stimulus: A and D, butanol; B, oil of cloves; C, methyl benzoate. Stimulus duration in A about 1.2 sec, in B and C about 1.5 sec. Record D was obtained with slower film speed in order to illustrate constancy of response during repeated stimulation. Time mark in C and D 0.5 sec. Vertical bar in C 0.5 mV.

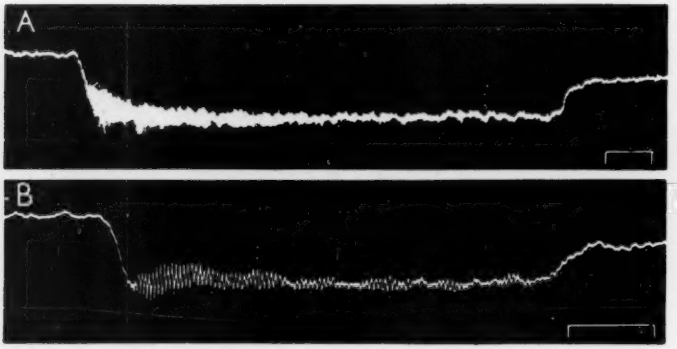

Fig. 3. Response of olfactory bulb evoked by passing a stream of odorized air through the nose. Stimulus: oil of cloves. Time mark in A 1 sec, in B 0.5 sec.

Further evidence of the slow adaptation of the olfactory bulb was provided by experiments with continuous stimulation. If a constant stream of odorized air was passed through the nose the bulb developed a persisting positivity (Fig. 3). The waves usually declined gradually during the stimulation. However, their behaviour varied greatly and sometimes they outlasted the sustained potential.

Due to the many uncertainties involved in stimulation no attempts were made to measure the relation between the magnitude of the response and the intensity of the stimulus. Pure air alone usually induced a small response. This could in many cases be traced to insufficient purification of the air or to contamination of the nose cannulae. The stimulus set-up was therefore arranged so that it could easily be cleaned. However, in most preparations a response was evoked although all possible precautions had been taken to avoid contamination. One possible explanation is that the air enclosed in the outer nasal passages contained odorous material that was brought to the olfactory region with the stream of pure air.

The response to odorized air was abolished when the *fila olfactoria* were cut or if the bulb was pinched. No response was produced by the forebrain; nor could any response be recorded from the contralateral bulb.

As seen in Fig. 4 A there is a close resemblance between the responses set up when the animal was made to inspire room air through the nostrils and those produced by blowing air into the nose with the pump. Although the air in the laboratory was felt odourless to the experimenter it acted as a strong stimulus to the rabbit's olfactory membrane. Purification of the air with activated carbon reduced the response but did not abolish it (B). The slower

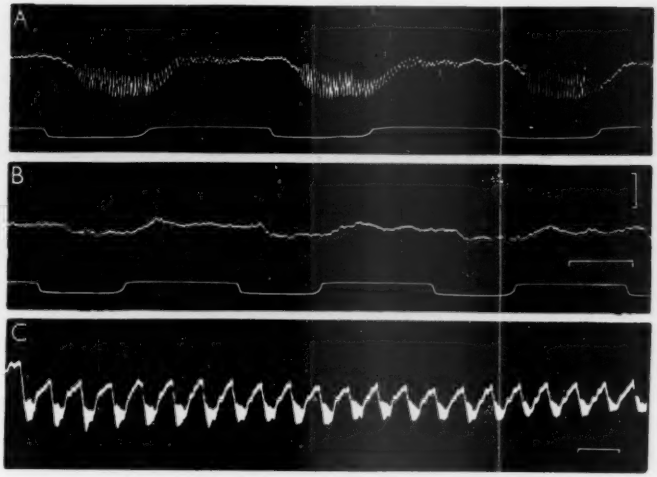

Fig. 4. Responses of olfactory bulb in rabbit breathing room air (A) and purified air (B). Respiration marked on lower beam. Record C, from another preparation, shows responses to room air in normal breathing rabbit. Time mark in B 0.5 sec, in C 2 sec. Vertical bar in B 1 mV.

record in C, illustrating the regular appearance of the responses, gives another example of the slow adaptation in the bulb.

The progressive action of urethane on the various components of the bulb response is illustrated in Fig. 5. As the anaesthesia was gradually deepened the intrinsic irregular activity vanished. The induced waves were less susceptible to the action of the drug and declined more slowly. At a level of anaesthesia

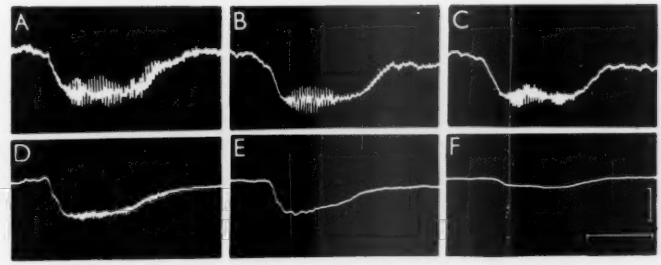

Fig. 5. Effect of gradually increasing depth of urethane anaesthesia. Stimulus: oil of cloves. Time mark 0.5 sec. Vertical bar 0.5 mV.

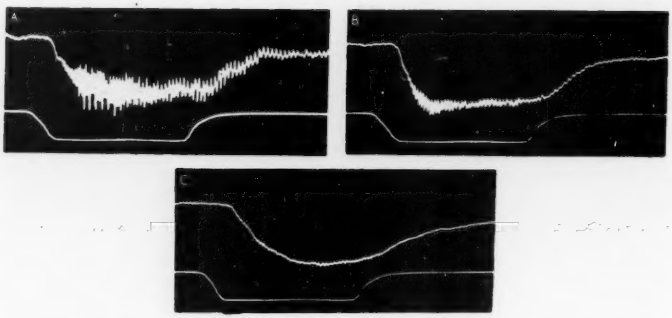

Fig. 6. Blocking of induced waves. A, control before, B and C during progressive action of topically applied cocaine solution (0.5 per cent). Stimulus: butanol. Time mark 0.5 sec. Vertical bar 0.5 mV.

at which the waves were abolished the slow potential was, however, only slightly reduced and remained as the only sign of activity in the completely silent bulb. Further deepening of the anaesthesia led to abolition of the response (F).

Asphyxia produces a similar sequence of changes in the bulb response as anaesthetics. In these experiments the animal was curarized and maintained on artificial respiration. As the respiration pump was stopped the spontaneous activity first increased and then declined and disappeared. The induced waves were depressed but could still be evoked after the intrinsic activity had vanished. The slow response was most resistant and did not vanish until after about 20—30 min of asphyxia.

The greater susceptibility of the induced waves to the action of anaesthetics made it possible to isolate the slow potential. Thus, it was found that topical application of cocaine (0.5 per cent) depressed (Fig. 6 B) and finally abolished (C) the waves without exerting any appreciable action on the slow potential. If the drug was washed away with Ringer's solution the waves slowly returned but usually did not regain their original height.

B. The slow response of the receptor organ

Recordings of the electrotonically conducted response. The olfactory nerve fasciculi emerge into the cranial cavity through numerous small canals in the *ethmoid* bone. Since the bundles have a length of only a few mm it is extremely difficult to expose them by dissection and record the fibre activity. However, if the bulb is removed and the electrode placed at the cut end of one of the bundles a slow response can be recorded when odorized air is blown into the nose (Fig. 7). In shape and time course this potential closely resembles the

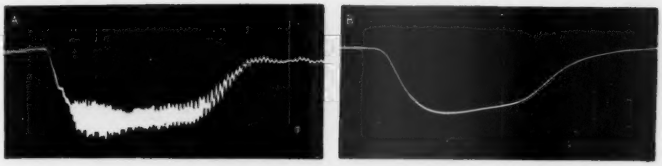

Fig. 7. Bulb response (A) and electrotonically conducted receptor potential (B), the latter recorded from a bundle of olfactory nerve fibres. Stimulus: butanol. Time mark 0.5 sec. Vertical bar 0.5 mV.

slow bulb response and behaves in the same way during various experimental conditions. In one respect the two responses react in a strikingly different way. As described above the slow bulb potential was abolished within 20—30 min of asphyxia. The potential recorded from a nerve bundle on the contrast was not affected by arrest of circulation (Fig. 8). This response was therefore at first assumed to be an artefact. However, the potential was found to increase with increasing stimulus strength and, furthermore, it was blocked with ether and reappeared after a while when the nose had been cleaned from the ether vapour. It was thus evident that the potential was not produced by some mechanical event during the passage of the air stream through the nose but that it had something to do with the excitation of the olfactory end organs.

The most probable explanation appeared to be that the response represented the receptor potential that was electrotonically conducted along the olfactory nerve fibres. But still it remained to be explained why the response could be evoked several hours after arrest of circulation. In previously reported experiments in the frog evidence was found supporting the view that the slow receptor potential is produced in the outermost layer of the olfactory epithelium. It can be assumed that under normal conditions this layer is supplied to a great

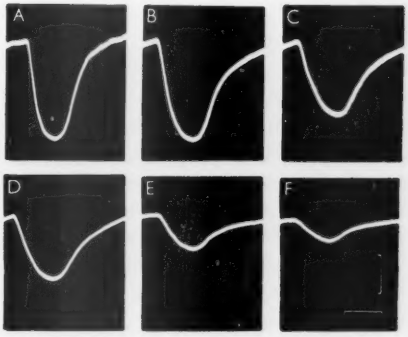

Fig. 8. Effect of asphyxia upon the electrotonically conducted receptor potential. A, control before; B—F, 1, 2, 3, 4 and 5 hours respectively after the artificial respiration had been stopped. Stimulus: butanol. Time mark 1 sec. Vertical bar 0.4 mV.

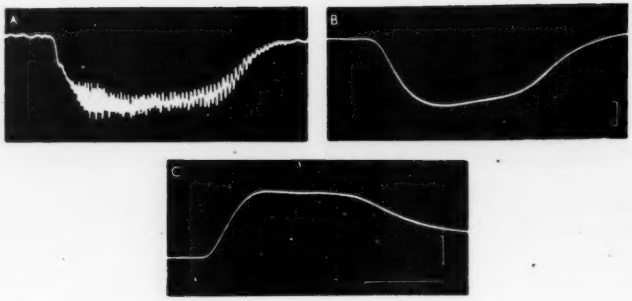

Fig. 9. Comparison of responses recorded from the olfactory bulb (A), the olfactory nerve bundles (B) and the olfactory mucosa (C). See text. Stimulus: butanol. Time mark 0.5 sec. Vertical bar in B 0.5 mV, in C 1 mV.

extent with oxygen taken up from the air in the nasal cavities. According to this hypothesis arrest of breathing and circulation should not induce anoxia unless the air enclosed in the nasal cavities is replaced by nitrogen. This was done in some experiments. No conclusion could, however, be drawn from these experiments since the results varied greatly, apparently because air was trapped in pockets in the nasal passages. In later experiments in the frog where the experimental conditions could be better controlled the olfactory mucosa was found to be remarkably resistant to anoxia.

Recordings from the mucosa. In order to determine the properties of the potential in the nasal mucosa it was desirable to record the response directly from the receptor layer. The experimental conditions are, however, very unfavourable when the olfactory mucosa is exposed, as bleeding and increased secretion are apt to occur. Incidentally another method was found that allowed recordings to be made from the olfactory mucosa without opening the nasal cavity.

In one of the experiments where recording was made from the cut end of an olfactory nerve bundle the electrode was unintentionally pushed through the canal in the cribriform plate into the nasal cavity. When olfactory stimulation was applied as usual by blowing air into the nose a response appeared that was of opposite sign to that obtained from the cut end of the nerve bundle. This negative potential was considerably larger than the positive one in the nerve but apart from these differences the two responses were almost identical. Evidently the electrode had been inserted into the sensory epithelium. The response consequently represented the directly recorded receptor potential.

In the subsequent experiments this potential was studied with the electrode inserted through the holes in the *ethmoid* bone into the olfactory epithelium. By this method it was not necessary to open the nasal cavity and the mucosa was left untouched.

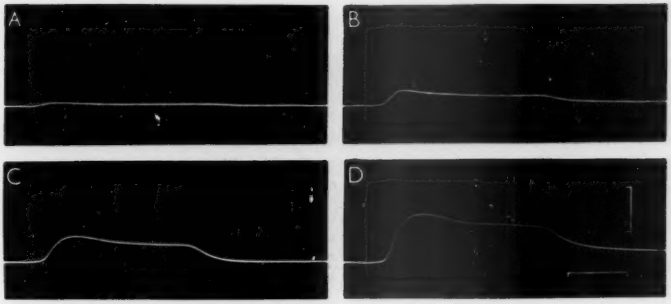

Fig. 10. Increase of the mucosa response with increasing stimulus strength. A, purified air; B, 0.001 M; C, 0.01 M; D, 0.1 M butanol. In this experiment stimulation was obtained by passing a constant stream of odorized air through the nose in order to study the rate of adaptation at various stimulus intensities. Duration of stimulation about 1.2 sec. Time mark 0.5 sec. Vertical bar 2 mV.

The records in Fig. 9 illustrate the typical sequence of events in the course of one experiment in this series. Recording was at first made from the dorsal surface of the bulb (A). After removal of the bulb the electrode was placed at the cut end of one of the nerve bundles and the response in B recorded. The electrode was then pushed through the nerve channel into the nasal mucosa. As stimulation was applied again the response in C appeared.

The response was not produced when the nasal mucosa of the opposite side was stimulated, nor did pure air set up more than an insignificant potential change.

Examination of the nasal response showed that in all essentials it behaved as the electro-olfactogram in the frog. Since this latter response has been described in detail (Ottoson 1956), the properties of the rabbit's olfactory receptor potential will only be briefly outlined in the following.

As mentioned above, it was very difficult to measure the relationship between stimulus strength and height of response with accuracy due to the difficulties encountered in controlling the parameters of the stimulus. To determine the validity of the results obtained the following criteria were used: 1) that pure air produced but an insignificant response 2) that the response to a given stimulus was constant when tested before and after a series of stimulations of different intensities. The responses in one of the experiments in which these criteria were fulfilled are illustrated in Fig. 10. A series of similar observations suggests that the relation between the size of the response and the intensity of the stimulus is the same as in the frog (Ottoson 1956).

Since the slow potential of the mucosa provided an opportunity for a direct study of the behaviour of the receptors it appeared to be of interest to examine

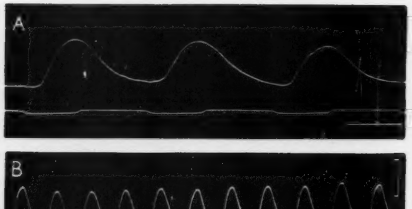

Fig. 11. Response of olfactory mucosa in rabbit breathing room air. The slower record in B illustrates regularity of response. Respiration marked on lower beam. Time marks 1 sec. Vertical bar 0.6 mV.

their ability to respond to intermittent and constant stimulation. Fig. 11 shows the responses recorded from the mucosa when the rabbit was breathing room air. The remarkable regularity in the shape of the potentials is obvious in the slower record B. Again, it may be noted that there is almost no reduction in amplitude. This could be predicted from the behaviour of the bulb response and is a definite evidence of ADRIAN's (1950) conclusion that there is no failure of the receptors under intermittent stimulation.

Further evidence of the slow adaptation in the olfactory end organ is provided by experiments in which a constant stream of odorized air was passed through the nose. Fig. 12 shows the responses obtained by stimulations of different duration. A stimulus lasting for 0.5 sec (A) gives rise to a potential with a steep rising phase, a rounded peak and an exponential fall towards base line. As the duration is increased potentials are produced with a plateau phase as seen in B and C. At the same time the falling phase is progressively slowed. The responses are almost identical with those obtained in previous experiments in the frog (OTTOSON 1956).

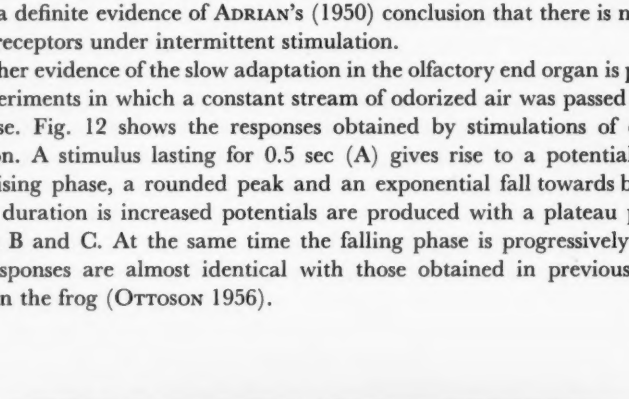

Fig. 12. Adaptation of olfactory mucosa. Stimulation with a constant stream of odorized air. Stimulus: oil of cloves. Duration of stimulation: A, 0.5 sec; B, 1 sec; C, 3 sec. Time mark in C 0.5 sec. Vertical bar 1 mV.

Discussion

The general properties of the slow potential change in the bulb suggest that it is homologous with the dendritic potentials of the cerebral cortex (ADRIAN 1936, CHANG 1951, CLARE and BISHOP 1955, PURPURA and GRUNDFEST 1956). The olfactory bulb is provided with an extremely well developed feltwork of fine nerve terminals in the glomerular layer and it is therefore scarcely surprising to find that a slow potential change is predominant in the reactions evoked in the bulb by natural stimulation.

It has been pointed out above that the potential developed in the foremost part of the bulb was negative while that recorded from the dorsal surface was positive. Histological sections of the bulb show that there is an aggregation of glomeruli in the anterior and ventral part of the bulb. This seems to account for the different signs of the potential in accordance with the theory of current spread in volume conductors.

It has been suggested by ADRIAN (1950) that the induced waves come from the dendrites of mitral cells. The dissociation of the slow potential and the waves by means of drugs or asphyxia indicates that these potentials arise in different structures. The present results support the view that the waves come from postsynaptic structures but do not give any clue as to their precise origin. As will be reported in a following paper observations in the frog provided definite evidence that the waves are produced by neurons of secondary order.

The slow bulb response is strikingly similar in shape and time course to the potential change in the mucosa. In an earlier investigation (OTTOSON 1954) the unwarranted conclusion was therefore drawn that the bulb potential represented the electrotonically conducted receptor potential. The later results clearly showed the differential origin of the two phenomena. However, this does not exclude the possibility that the receptor potential actually spreads to the bulb. The conspicuous magnitude of the response recorded from the cut ends of the nerve bundles as they enter into the cranial cavity strongly supports this view. It can be predicted that also the bulb potential spreads along the nerve fibres. Investigations on this point were, however, rendered impossible by the anatomical conditions. As will be reported in a later paper the spread of the bulb potential in the olfactory nerve could be examined in detail in the frog.

The experiments with repetitive stimulation clearly demonstrate the slow rate of adaptation in the olfactory end organs. It may therefore be assumed that under normal conditions each inspiration gives rise to an inflow of olfactory impulses to the brain. Within the bulb the structure producing the slow potential closely reproduces the excitatory processes in the sense organ. This does, however, not imply that the olfactory signals are transmitted without being modified. It has been shown by ADRIAN (1950) that the intrinsic ac-

tivity of the bulb interferes with the afferent signals. Further it has to be taken into account that the impulse transmission to rhinencephalic areas is influenced by centrifugal fibres (KERR and HAGBARTH 1955).

This work has been supported by grants from The Swedish Medical Research Council.

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Comparison of Slow Potentials Evoked in the Frog's Nasal Mucosa and Olfactory Bulb by Natural Stimulation

By

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Received 31 March 1959

Abstract

Ottoson, D. Comparison of slow potentials evoked in the frog's nasal mucosa and olfactory bulb by natural stimulation. *Acta physiol. scand.* 1959. *47.* 149—159. — A comparative study has been made of the slow potential changes induced in the frog's olfactory bulb and nasal mucosa. It was found that the bulb response closely reproduced the slow potential changes in the receptor organ. Thus, changes in stimulus strength brought about identical changes in height of the two potentials. The response of the bulb was sensitive to asphyxia while the receptor response remained unaltered for hours after arrest of circulation. Antidromic stimulation of secondary olfactory pathways blocked the induced waves of the bulb but left the slow potential unaffected. Strong stimulation sometimes gave rise to oscillations superimposed upon the receptor potential. The frequency of the bulbar waves was not influenced by these oscillations. When the olfactory nerve was stimulated antidromically a diphasic spike potential could be recorded from the nasal mucosa. This potential exhibited the same distribution pattern as the receptor response. Repetitive maximal antidromic stimulation of the nerve did not affect the response evoked in the mucosa by natural stimulation.

In previously reported experiments in the rabbit it was shown (Ottoson 1954, 1959) that the olfactory bulb develops a sustained potential when the nose is stimulated with odorized air. In the course of the analysis of the events

leading to the production of this potential it was found essential to make simultaneous recordings from the bulb and the receptor organ. This is difficult to do in the rabbit as in most mammals because of the inaccessibility of the olfactory fibres and end organs. The frog's olfactory system provides, however, favourable anatomical opportunities for such recordings.

The present paper gives an account of experiments carried out on frogs with the aim to obtain information about the correlation between the receptor potential and the slow bulb response. A comparative analysis was made of the behaviour of the two responses under various experimental conditions.

Methods

All experiments in the present investigation were carried out on frogs (*Rana temporaria*). Curare (0.1—0.15 cc tubocurarine *Vitrum*) was injected into the dorsal lymph sac and the skin over the head anaesthetized with lidocaine (1 per cent). The forebrain was then exposed and covered with mineral oil. Access to the olfactory epithelium was gained by cutting off the dorsal wall of the nasal cavity.

The potentials of the olfactory bulb were recorded with electrodes of the usual agar—AgCl-Ag type (tip diam. 0.1 mm). The reference electrode was placed at the occipital bone and the preparation grounded through a chlorided silver-electrode. The potentials were amplified with a differential direct coupled amplifier (HAAPANEN 1953) and recorded on an oscilloscope (Tektronix 535).

Olfactory stimulation was accomplished by blowing a small amount of odorized air into the nasal cavity. Butanol was used as stimulating agent in all experiments.

In all records an upward deflection represents a negative potential at the tip of the recording electrode.

Results

A. Characteristic properties of the bulb response in the frog

As described by GERARD and YOUNG in 1937 the olfactory bulb in the frog shows greater spontaneous activity than other parts of the brain. An electrode placed at the surface of the bulb usually records irregular waves at 4—10 a second but sometimes regular waves are obtained. The pattern of activity varies from one preparation to another and also for different recording sites. Generally the entrance zone of the olfactory nerve exhibits less activity than other parts of the bulb. The asynchronous spontaneous activity is not maintained by the inflow of afferent impulses since the rhythm remains unaltered after cutting of the olfactory nerves.

As illustrated in Fig. 1 natural stimulation of the olfactory mucosa gives rise to a slow potential change in the bulb. Superimposed upon this potential there are regular oscillations at 8—12 per sec. When the stimulus strength is increased the slow potential increases in magnitude up to a maximum, the induced waves become larger and their frequency is increased. When weak stimuli are used (A) the irregular intrinsic activity is almost unaffected in the

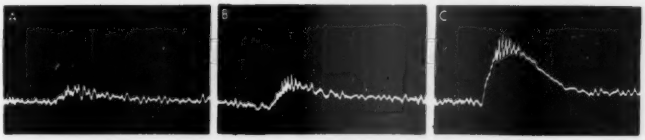

Fig. 1. Bulb responses induced by natural stimulation of the nasal mucosa. A, 0.001 M; B, 0.01 M; C, 0.1 M butanol. Time mark 2 sec. Vertical bar 1 mV.

course of the slow potential change. With strong stimulation the picture is dominated by the induced waves and when they vanish a silent period follows during which there is no spontaneous activity (C).

Distribution of the response. Exploration of the bulb with the recording electrode revealed that the response varied widely in shape from one recording site to another. Potentials with the same features as the response seen in Fig. 1 C were obtained only from the rostral part of the bulb. As the electrode was moved towards the cerebral hemisphere the potential became successively smaller and was often preceded by a positive deflection (Fig. 2 E). When the recording was made from regions close to the cerebral hemisphere a small

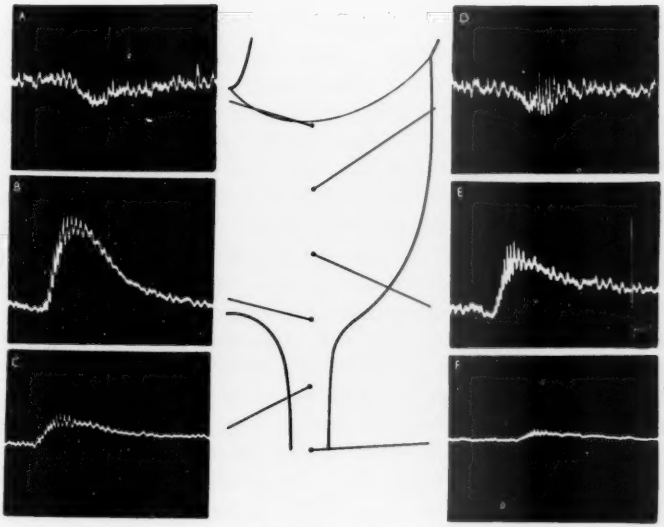

Fig. 2. Distribution pattern of responses induced in olfactory bulb by stimulation of nasal mucosa with butanol (0.01 M). See text.

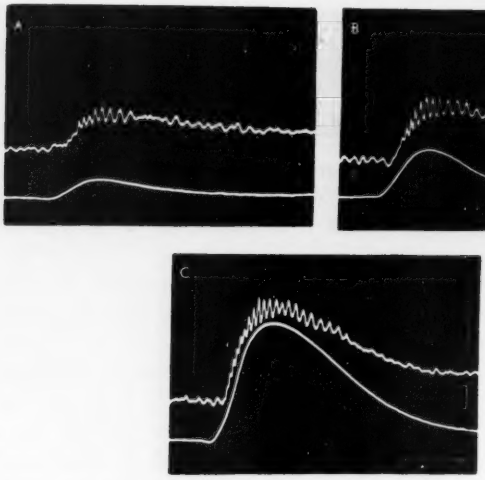

Fig. 3. Comparison of increase of EOG (lower trace) and bulb response (upper trace) with increasing stimulus strength. A, 0.001 M; B, 0.01 M; C, 0.1 M butanol. Time mark 1 sec. Vertical bars 1 mV; the upper bar relates to the bulb responses, the lower one to the EOG.

response of positive sign was obtained (A, D in Fig. 2). Inbetween the region giving negative potentials and that giving positive ones it was often possible to find recording sites from which only induced waves could be obtained. As shown by records B, C and F in Fig. 2 the response spreads out in the olfactory nerve and changes in a manner typical of electrotonically propagated potentials. Thus, as the electrode is moved away from the bulb the potential decreases in magnitude and the rising phase is progressively slowed. Pinching of the bulb or the nerve abolishes the response.

The contralateral bulb also develops small responses with the same general characteristics as those described above. These responses appear to be produced in the bulb of the stimulated side and recorded due to simple electrical conduction. This conclusion is borne out by the observation that the responses were not abolished when the contralateral bulb was pinched but disappeared after pinching of the bulb of the stimulated side. However, the experiments do not allow any definite statement on this point. The possibility cannot be excluded that part of the response in the contralateral bulb was actually produced there. As shown by CAJAL (1922) there are connections between the two bulbs that would provide the anatomical basis for a spread of excitation.

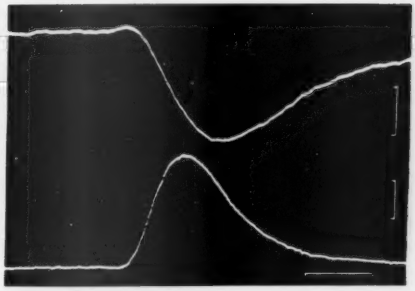

Fig. 4. Comparison of the electrotonically conducted potential in the olfactory nerve (upper trace) and the response obtained from the nasal mucosa (lower trace). Stimulus 0.01 M butanol. Time mark 1 sec. Vertical bars: upper one 0.1 mV; lower one 0.5 mV.

B. Comparison of the bulb response and the slow potential in the sensory epithelium

As described in detail in previous papers (Ottoson 1956, 1958) the olfactory membrane produces a negative potential when stimulated with odorized air. For the sake of convenience this response will in the following be called the electro-olfactogram (EOG). The records in Fig. 3 clearly show the striking resemblance between the EOG (lower trace) and the bulb response (upper trace). Of particular interest is the close correlation between the two potentials in terms of changes in height with increasing stimulus strength.

Pinching of the bulb leads to disappearance of the bulb response but has no effect on the EOG. This finding together with the above described decremental spread of the potential along the olfactory nerve (F in Fig. 2) demonstrates that the bulb response is not due to electrotonic propagation of the EOG or *vice versa*. An interpretation along these lines would, however, not be entirely unreasonable as the olfactory nerve is only 1—2 mm long. Since the bulb response spreads a considerable distance along the nerve the question arises if there is also in the frog a spread of the EOG corresponding to that observed in the rabbit (Ottoson 1959). The answer to this question is given in Fig. 4. In this case the olfactory bulb was removed and recording made from the cut end of the olfactory nerve. When the mucosa was stimulated with odorized air a slow potential could be recorded from the nerve. This potential was almost identical with the EOG but of opposite sign. The response was not picked up from the mucous membrane due to distant recording since it was not obtained after the nerve had been crushed. The obvious conclusion therefore appears to be that the response recorded from the olfactory nerve represents the electrotonically conducted receptor potential. The features of the potential in comparison with the directly recorded EOG also suggest this. The difference in polarity is explained by the recording conditions.

The effect of circulatory arrest. In experiments in the rabbit it was found that the bulb response was rapidly reduced while the EOG remained unaltered for

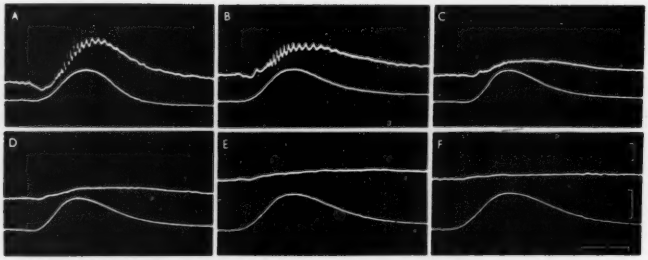

Fig. 5. Arrest of circulation. Comparative effect upon the EOG (lower trace) and upon the bulb response (upper trace). A, control before; B—F 10, 30, 60, 90 and 120 min respectively after the circulation was stopped. Stimulus: 0.1 M butanol. Time mark 1 sec. Vertical bars: upper one 0.5 mV; lower one 2 mV.

hours after the respiration was cut off. These experiments have been repeated in the present investigation on the frog where the effect upon the two responses could be followed with simultaneous recordings. The records in Fig. 5 illustrate the result of one of these experiments. In record A is shown the EOG and the

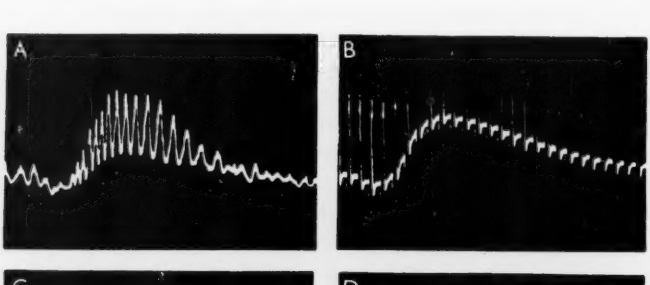

Fig. 6. Effect of antidromic stimulation upon bulb response (A and B). The spikes superimposed upon the slow potential in B consist of shock artifacts and the action potentials of antidromically activated structures. C and D, effect of antidromic stimulation of the olfactory nerve upon the slow receptor response. The superimposed spikes are the action potentials of the olfactory nerve. See text.

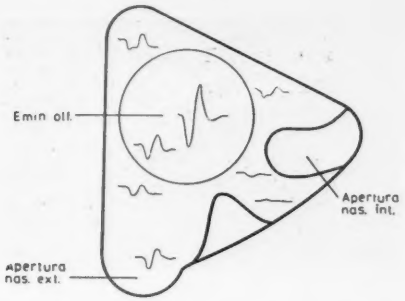

Fig. 7. Distribution pattern of antidromically evoked spike potentials in the nasal mucosa.

bulb response before the circulation was stopped. Records B—F were taken subsequently at regular intervals. It will be noted that the slow response of the bulb is gradually depressed but can still be elicited after the induced waves have been abolished (D). About two hours after arrest of the circulation the bulb failed to give any response (F). In striking contrast to the reduction and abolition of the bulb response the EOG remained almost unaffected. At the time when the bulb response had vanished the EOG was as large as at the beginning of the experiment. The only change that occurred was a slowing of the potential.

The effect of antidromic stimulation. The effect of antidromic stimulation of secondary neurons was studied in order to find out to what extent postsynaptic structures are involved in the production of the bulbar response. The antidromic stimulation was applied with two electrodes pushed into the brain close to the optic lobe. As will be described in a later paper antidromic stimulation blocks the second component of the bulbar response evoked by electrical stimulation of the olfactory mucosa. This finding was taken advantage of in the present study in order to ensure that the strength of the antidromic stimulus was appropriate.

Record B in Fig. 6 shows the effect of the antidromic stimulation. In this preparation olfactory stimulation alone produced a bulbar response with superimposed oscillations of considerable height (A). As seen in B these waves were completely blocked during antidromic stimulation while the slow potential remained unaltered. This effect suggests that the induced waves arise in postsynaptic structures. It further indicates that the slow potential is of presynaptic origin.

Responses evoked in the olfactory mucosa by antidromic stimulation of the olfactory nerve. If an electric shock is applied to the olfactory nerve the antidromic volley can be recorded from the surface of the mucosa as a diphasic spike potential. The response is obtained only from regions lined with sensory epithelium and varies in a typical manner from one recording site to another,

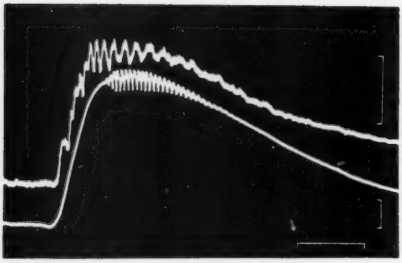

Fig. 8. Comparison of oscillatory potentials superimposed upon the EOG and the induced waves of the bulb. Stimulus: 0.1 M butanol. Time mark 1 sec. Vertical bars 1 mV.

as illustrated in Fig. 7. The interesting point is the great similarity between the distribution of the nerve action potential and the distribution of the EOG (Ottoson 1956). The fact that no response is obtained from areas with indifferent epithelium further shows that the olfactory nerve contains no other fibres than those carrying olfactory signals.

In earlier experiments (Ottoson 1956) it was observed that antidromic stimulation of the olfactory nerve does not influence the EOG. In these experiments the size of the nerve action potential recorded from the olfactory mucosa was not checked. The experiments have therefore been repeated in the present investigation. It was found that repetitive stimulation of the olfactory nerve with shocks sufficiently strong to evoke spikes of maximal height left the response to natural stimulation almost unaffected (Fig. 6 C and D). The fact that the EOG was not reduced although the olfactory fibres were thrown into maximal activity clearly shows that the slow response does not arise in the fibres.

Oscillations superimposed upon the EOG. Rhythmic waves at about 20 per sec sometimes appear superimposed upon the EOG. As a rule they are obtained only with strong stimulation and occur most often in preparations that exhibit signs of deterioration. These oscillations appeared in some of the experiments in which simultaneous recordings were made from the bulb and the mucosa. This provided ample opportunity for an analysis of the relationship between the waves in the bulb and the oscillations in the mucosa. Fig. 8 shows a record from one of these experiments. It may be observed that the oscillations in the EOG start later than the bulbar waves and outlast them. Further, the peripheral oscillations have a considerably higher frequency than the bulbar waves. These facts clearly reveal the mutual independence of the two events.

The oscillations in the EOG may either be produced by a synchronous activity within groups of receptors or by a corresponding activity in the olfactory nerve fibres (Adrian 1955, 1957). An observation relating to this question was incidentally made as the oscillations appeared in one of the experi-

ments with antidromic stimulation. It was observed that the antidromic impulses interfered with the waves. As the oscillations rapidly disappeared in this preparation and were not obtained in any of the following ones there was no opportunity to further examine this phenomenon. Various experimental procedures, for instance prolonged olfactory stimulation, were used in order to induce the oscillations but all these attempts were unsuccessful.

Discussion

The previous analysis of the production of the bulb response in the rabbit (Ottoson 1959) was greatly hampered by the difficulties encountered in simultaneous recording of the peripheral and central electrical phenomena induced by the olfactory stimulus. When frogs are used one does not meet with these difficulties. Another advantage of using frogs is that the stimulus can be better controlled than in the rabbit.

In the frog the peripheral and central activity changes could be followed by simultaneous recordings from the nasal mucosa and olfactory bulb. It was thus possible to make a comparative study of the characteristics of the responses at these two sites. The results clearly demonstrate the close correlation between the bulb potential and the EOG. The events in the sense organ thus appear to be faithfully reproduced centrally as far as the slow potential changes in the bulb are concerned.

The experimental evidence gained in the rabbit suggested that the slow bulb response is of synaptic nature. The results presented above strongly support this view. Because the entire bulb could easily be exposed in the frog it was possible to map out the distribution of the response. As shown in Fig. 2 the greatest negative responses were obtained at the entrance zone of the olfactory nerve while areas close to the cerebral hemisphere produced positive potentials of low amplitude. This finding is in accordance with what was found in the rabbit and can evidently be accounted for by the structural characteristics of the bulb. Although numerous investigations have been made on the morphology of the frog's brain the data on the development of the layers in different parts of the bulb are incomplete. Histological sections of the bulb were therefore examined in order to see if the distribution of the response could be correlated with the distribution of the glomeruli. In all bulbs that were examined it was found that the glomeruli were densely packed in the rostro-ventral region while they were more scattered in other parts. The accumulation of the glomeruli in the foremost part of the bulb is also seen in Fig. 9 from a paper of Cajal (1922).

Further evidence relating to the origin of the slow bulb response was obtained with antidromic stimulation. It has earlier been demonstrated by Kerr and Hagbarth (1955) that stimulation of the basal rhinencephalic area in the

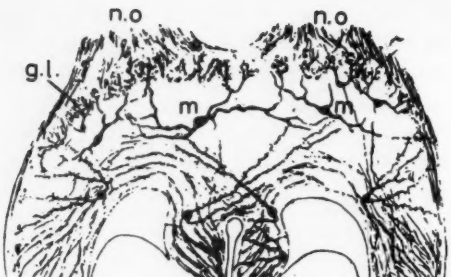

Fig. 9. Structure of olfactory bulb in the frog. Histological section showing: n. o., olfactory nerves, g.l. glomerular layer, m, mitral cells. From CAJAL (1922).

cat produces a depression of the induced waves. A similar effect was obtained in the frog, as shown in Fig. 6. No attempt was made to decide whether this effect was attributable to antidromic blocking or if it was exerted by centrifugal fibres. Whatever mechanism is operating the mere fact that the induced waves were blocked reveals that they can be ascribed to activity in secondary neurons. At the present stage of investigation it is not possible to decide if or to what extent postsynaptic structures are involved in the production of the slow bulb response. The observations on the effect of antidromic stimulation speak in favour of a presynaptic origin but do not exclude that also postsynaptic neurons participate in the generation of the response.

The data presented above suggest that the basic events involved in the initiation and transmission of olfactory signals are the following. When the nasal mucosa is stimulated with odorous substances the olfactory end organs develop a sustained potential that spreads along the fibres and gives rise to an impulse discharge. The afferent inflow into the bulb results in the production of a slow potential change in the glomeruli. This potential closely follows the changes in the potential produced by the receptors. Thus it increases in parallel with the receptor potential and as it becomes larger an increasing number of secondary neurons are thrown into synchronous activity. In gross recordings this synchronous activity is reflected as regular waves superimposed upon the slow potential. The waves are accompanied by bursts of impulses in axons passing centrally to rhinencephalic centers (ADRIAN 1950).

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Olfactory Bulb Potentials Induced by Electrical Stimulation of the Nasal Mucosa in the Frog

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Abstract

Ottoson, D. Olfactory bulb potentials induced by electrical stimulation of the nasal mucosa in the frog. *Acta physiol. scand.* 1959. *47.* 160—172. — The potential changes induced in the frog's olfactory nerve and bulb by electrical stimulation of the nasal mucosa have been studied. The response developed by the bulb is composed of two successive negative deflections. It has been possible to segregate these deflections and partly identify their origins. The first component of the response is of synaptic nature and builds up a persisting potential during repetitive stimulation. The second component of the response is sensitive to the action of narcotics and asphyxia and is blocked when secondary neurons are stimulated antidromically. The conclusion has been drawn that this component represents the propagated activity in neurons of secondary order. The properties of the olfactory nerve fibres have been studied in recordings of the nerve action potential.

The previously reported investigations (Ottoson 1959 a, b) were mainly concerned with the production and nature of the potentials evoked in the bulb by olfactory stimulation. It was felt that more could be learned particularly about the synaptic transmission of olfactory signals by using electrical stimulation instead of natural. Such an investigation appeared to be of interest not only for the evaluation of olfactory functions. The olfactory bulb has remained essentially unchanged during the phylogenetical development of

the brain and manifests certain specific morphological features. Thus, the afferent system is uniform and the synaptic transmission to secondary neurons occurs within a well defined layer of fine nerve terminals and dendrites. The analysis of the bulb potentials might therefore be expected to give data of general neurophysiological interest.

Until quite recently there have been no reports on studies of responses set up in the bulb by electrical stimulation of the afferent fibres. In an investigation on the pyriform responses MAC LEAN, ROSNER, and ROBINSON (1957) observed that electrical stimulation of the *fila olfactoria* in the opossum gave rise to a slow potential change in the bulb but they did not further analyse this response.

The present report deals with observations on the characteristics of the olfactory bulb response in the frog. Attempts have been made to isolate the main components of the response and locate their origins. An account will also be given of observations on the properties of the olfactory nerve.

Methods

The experimental procedure has been described in detail in a previous paper (OTTOSON 1959 b).

To stimulate the nasal mucosa pulses of 0.2 msec duration were applied through Pt-electrodes. When stimulation of a small area was desired a pair of needle electrodes were employed with the tips less than 0.5 mm apart.

All recordings of the bulb responses were made with a differential direct coupled amplifier (HAAPANEN 1953) while the olfactory nerve action potentials were recorded with a condenser coupled amplifier with a time constant of 1 sec.

Results

A. The response produced in the olfactory bulb by electrical stimulation of the nasal mucosa

General characteristics. A synchronous volley of afferent impulses evoked by electrical stimulation of the nasal mucosa gives rise to a negative potential change in the olfactory bulb (Fig. 1). The response has a duration of 100—200 msec and is purely monophasic at weak or intermediate stimulus strength. Strong stimulation usually sets up potentials with a late positivity (C). As the strength of the stimulus is increased the peak of the response moves earlier while the latency remains unchanged (D). Of particular interest is that the invasion of the bulb occurs in two stages, as revealed by the slight inflexion that interrupts the rising phase of the response.

Distribution pattern. The responses illustrated in Fig. 1 were obtained with the electrode placed at the surface of the olfactory bulb close to the entrance of the olfactory nerve. Mapping of the distribution of the potential established

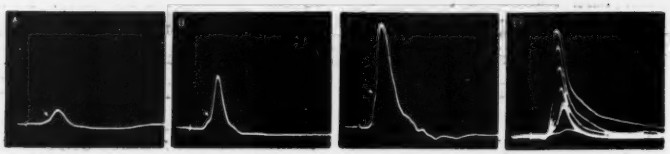

Fig. 1. The action potential of the olfactory bulb. A—C, responses to electrical shocks of increasing strength. D, superimposed records from another preparation. Time mark 100 msec. Vertical bar 1 mV.

that different parts of the bulb developed responses of varying size and configuration. As the electrode was moved away from the rostral zone in caudal direction the response became rapidly smaller and there appeared an initial positive dip. The results obtained by exploring the bulb surface with the electrode were in striking accordance with those in earlier experiments with natural stimulation and will therefore not be dealt with further.

Stimulation of different areas in the nasal mucosa. The distribution of olfactory receptors shows great variations in different parts of the nasal mucosa (GAUPP 1904). They are usually densely packed in the epithelium lining *eminentia olfactoria.* In other regions they are more scattered and to a varying extent replaced by indifferent epithelial cells.

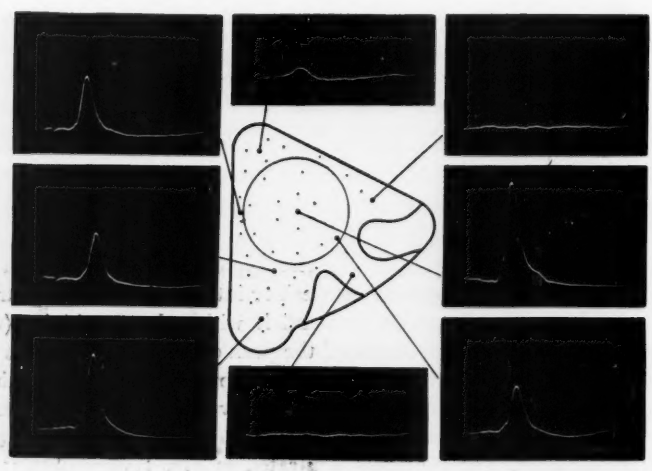

Fig. 2. Responses set up in the olfactory bulb by stimulation of various regions of the nasal mucosa. Dotted area marks sensory epithelium. Time mark 100 msec. Vertical bar 1 mV.

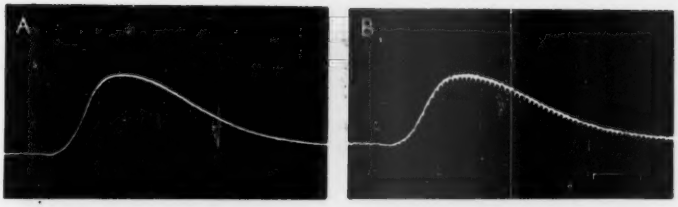

Fig. 3. Electrical inexcitability of the olfactory end organs. A, response of olfactory epithelium to stimulation with butanol. B, response to the same stimulus during electrical stimulation of the mucosa at 10/sec. Stimulus artefacts superimposed upon the slow potential. Time mark 1 sec.

Punctate stimulation was applied in order to see to what extent various parts of the mucosa contribute to the production of the bulb response. Weak stimuli were used in order to obtain stimulation of small areas only. Fig. 2 illustrates a series of observations made in one of these experiments. It may be noted that responses could be evoked in the bulb only when the sensory epithelium was stimulated. Thus, the stimulus was ineffective when the stimulating electrode was moved to the lateral recess where the mucosa lacks olfactory end organs. This observation is of interest since it proves that the potential developed in the bulb arises as a result of stimulation of olfactory endings. It has been reported (DOGIEL 1887) that the unmedullated olfactory fibres in the olfactory nerve are intermingled with myelinated axons. It could therefore not *a priori* be excluded that the potential recorded from the bulb partly came from other structures than olfactory ones. The close correlation between the distribution of olfactory endings and the potential pattern in the nasal mucosa shows that this is not the case.

Electrical excitability of the end organs. In the course of these experiments the question arose whether the electrical stimulus excited the olfactory fibres in

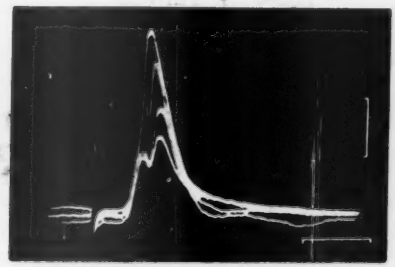

Fig. 4. Effect of repetitive stimulation upon the response of the olfactory bulb. Superimposed records of responses set up by stimulation at 1/sec. Time mark 100 msec. Vertical bar 1 mV.

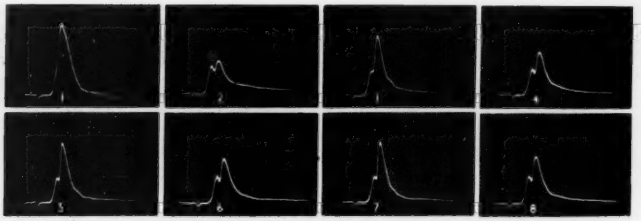

Fig. 5. Records of consecutive bulb responses (1—8) to stimulation of the olfactory mucosa at 1/sec.

the mucosa directly or via the end organs. In order to get an answer to this question the following experiment was carried out. Stimulating electrodes with tips 1 mm apart were placed in contact with the epithelium on *eminentia olfactoria*. A nonpolarizable recording electrode was then brought in contact with the mucosa between the tips of the stimulating electrodes and the response to natural stimulation was recorded (Fig. 3 A). The mucosa was then stimulated with electrical shocks at 10/sec and the natural stimulation applied again. Record B in Fig. 3 illustrates that the receptor potential remained almost unchanged. The conclusion would therefore be that the olfactory fibres are directly excited by the electrical shocks.

The properties of the components of the bulb response. The presence of the inflexion on the rising phase suggests that the response is built up of two main components. This conclusion is borne out by the effect produced by repetitive stimulation. Thus, it was found that stimulation at frequencies above 1/sec caused a pronounced reduction of the potential and a segregation into two phases, hence-

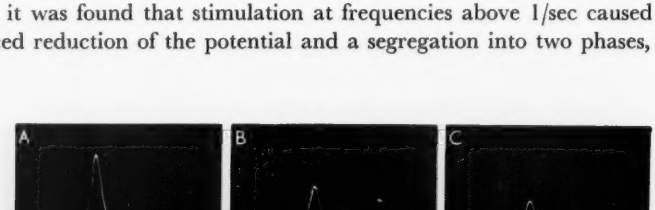

Fig. 6. Progressive action of nembutal upon response of the olfactory bulb. A, before; B—F, 15, 30, 60, 80 and 90 minutes respectively after 1 cc nembutal (1 : 500) had been injected intraperitoneally. Time mark 100 msec. Vertical bar 1 mV.

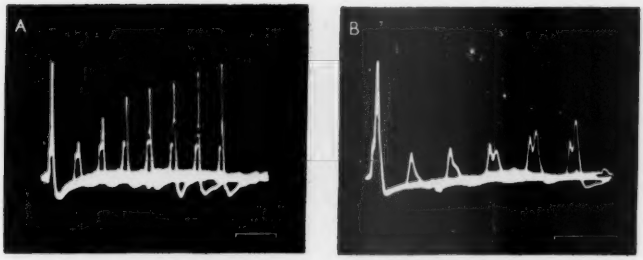

Fig. 7. Time course of recovery of the two phases of the bulb response. Time mark in A 500 msec, in B (another preparation) 250 msec.

forth called p_1 and p_2. It is quite clearly seen in Fig. 4 that p_1 is not affected during repetitive stimulation while p_2 is greatly diminished.

Usually p_2 was progressively reduced but in some experiments there occurred a regular alternation in height. This is illustrated in Fig. 5 which shows the consecutive responses to stimulation at 1/sec.

An effect similar to that caused by repetitive stimulation was obtained when natural stimulation was applied slightly before or simultaneously with the electrical shock. In this case, however, p_1 as well as p_2 were depressed. The recovery of the bulb after natural stimulation could be followed by applying electrical test shocks at increasing intervals after the olfactory stimulus. However, it was apparent that a more precise determination of the excitability changes in the bulb could be made if electrical shocks were used as conditioning stimuli.

The different properties of p_1 and p_2 were also demonstrated with anaesthetics and asphyxia. The main effects of nembutal injected intraperitoneally was a depression of p_1 and p_2 and a delay of p_2 (Fig. 6). Since p_2 was the most vulnerable component, as revealed by its rapid decay, p_1 was finally left behind almost pure. The same sequence of blocking was obtained with asphyxia.

The foregoing experiments clearly demonstrate the existence of two components, but with none of the experimental procedures used was it possible to abolish p_2 without causing a simultaneous change in p_1. An isolation of an unaltered p_1 was, however, achieved by applying two shocks with suitably timed intervals (Fig. 7). When two successive shocks were applied to the mucosa the stimulus interval had to be kept at about 2—3 sec in order to obtain a full-sized response to the second stimulus. As the stimulus interval was made successively shorter the response gradually decreased. In A it is seen that this decrease was entirely due to a reduction of p_2 while p_1 remained unchanged in height. At a critical stimulus interval of about 0.3 sec p_2 failed to develop and p_1 was obtained alone. Further reduction of the stimulus interval was

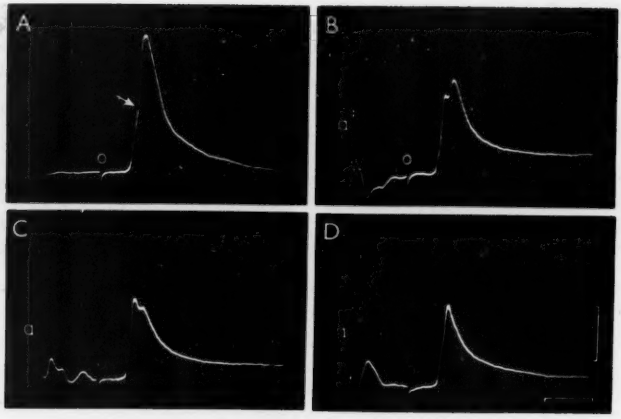

Fig. 8. Blocking effect of antidromic stimulation. A, the bulb response to ortodromic stimulation alone. B—D, ortodromic stimulus (o) preceded by antidromic stimulation (a) of increasing strength. The antidromic stimulus was applied with two electrodes inserted into the brain rostral to the optic lobe. Initial phase of the ortodromic response is not affected while the second phase is blocked. Time mark 100 msec. Vertical bar 1 mV.

not followed by any decrease of p_1 until the second stimulus was applied less than 200 msec after the first. As will be described later the olfactory nerve has a comparatively long recovery period. The diminution of p_1 at stimulus intervals shorter than 200 msec was therefore not due to any failure of the bulbar structures to produce a full-sized p_1 but could be ascribed to the reduced afferent input to the bulb.

Effect of antidromic stimulation. Antidromic stimulation of secondary olfactory pathways was found to offer another possibility to obtain an isolated p_1. The antidromic shocks were delivered through two needle electrodes inserted into the brain slightly rostral to the optic lobe. The majority of secondary tracts appears to end in these regions (see CAJAL, 1922). For obvious reasons it was not possible to obtain a selective stimulation of secondary olfactory neurons and a widespread effect was induced. For the purpose of the actual experiments this effect was negligible.

A single antidromic shock induced a complicated series of potential changes in the bulb. If an ortodromic shock was applied shortly after the antidromic stimulus the bulb was not capable to produce a full-sized response. Record B in Fig. 8 illustrates that the antidromic stimulation caused a pronounced reduction of p_2 but did not affect p_1. Gradual increase of the antidromic stimulus strength caused a progressive depression and finally an abolition of p_2. In striking contrast p_1 remained entirely unaltered. In order to examine whether

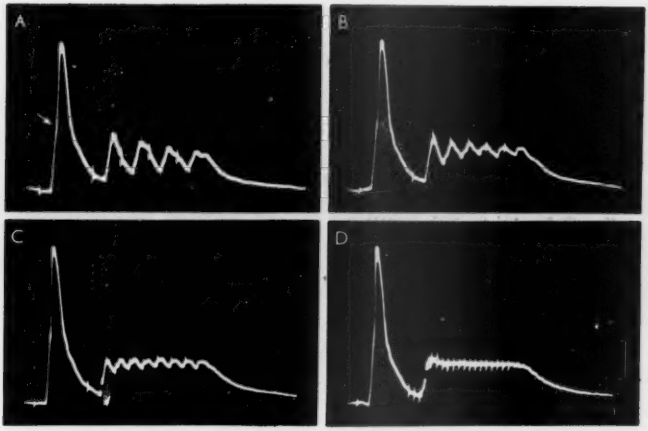

Fig. 9. Summation of initial phase of the bulb response. Frequencies of repetitive stimuli in the test volleys: A, 10; B, 15; C, 20; D, 50/sec. Time mark 200 msec. Vertical bar 1 mV.

or not the different action on p_1 and p_2 was simply a matter of difference in threshold the strength of the antidromic stimulus was further increased. However, no effect on p_1 was achieved even with an antidromic stimulus twice as strong as that needed to abolish p_2.

Summation of p_1. The nature of p_1 was further elucidated in experiments with a train of shocks following upon a conditioning stimulus. The interval between the conditioning stimulus and the test volley was kept shorter than the absolute refractory period of p_2 in order to obtain p_1 alone. Fig. 9 A illustrates that each shock in the test volley gives a distinct response at low frequency of stimulation. As the stimulus frequency is increased the responses fuse to a sustained potential with a smooth plateau phase followed by a slow decay towards base line at the end of the stimulation period.

Occlusion. In the analysis of potentials in the central nervous system occlusion has generally been accepted as a criterion of postsynaptic origin. The experi-

Fig. 10. Occlusion. A and B olfactory bulb potentials elicited by stimulation of two separate areas of the nasal mucosa. C, the two stimuli applied together and timed so that the afferent volleys enter the bulb simultaneously. Time mark 100 msec. Vertical bar 1 mV.

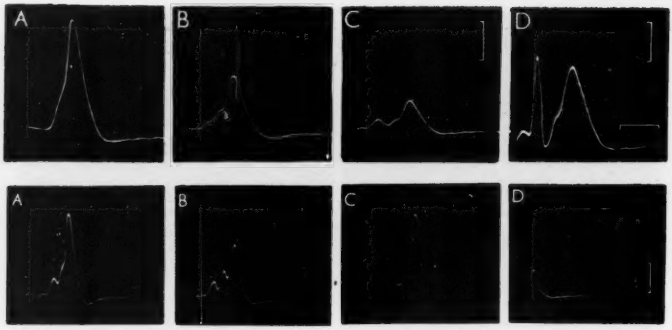

Fig. 11. Upper row: decremental spread of bulb potential in the olfactory nerve. Responses recorded at increasing distances from the bulb: A, at the entrance of the nerve; B, 0.7; C, 1.4; D, 1.8 mm from the bulb. Time mark 100 msec. Vertical bar in C 1 mV, in D 0.3 mV. Lower row: A and B, segregation of the components of the response by repetitive stimulation at 1/sec. C and D, another preparation, C before, D after pinching of the bulb. Time mark 100 msec. Vertical bar 1 mV.

mental results presented above clearly showed that p_2 came from postsynaptic structures. With regard to the origin of p_1 no definite inference could be made. Experiments were therefore carried out to see whether or not p_1 occluded. Punctate stimulation was applied to different parts of the nasal mucosa by using two pairs of electrodes. One of these was usually placed at the mucosa of *eminentia olfactoria* and the other in *vestibulum nasi*. Records A and B in Fig. 10 show the responses obtained from the bulb when these areas were stimulated separately. The potentials have different latencies owing to the different conduction distances. When occlusion was tested the stimuli had therefore to be timed in such a way as to cancel the latency difference. When so applied the two stimuli gave rise to a response that was less than the sum of the individual potentials. A close examination of the records reveals that p_1 as well as p_2 occluded. The occlusion of p_2 was anticipated from the foregoing experiments, particularly from those with antidromic stimulation. The fact that p_1 occludes indicates that also this potential or a fraction of it arises in postsynaptic structures.

B. Potentials recorded from the olfactory nerve

Electrotonical conduction of the bulb response. From the previously reported experiments with natural stimulation it was known that the electrotonically conducted bulb response overshadows all other electrical signs of activity in the nerve. It was therefore not surprising to find that the same holds true for the electrically induced responses. The spatial decrement of the potential

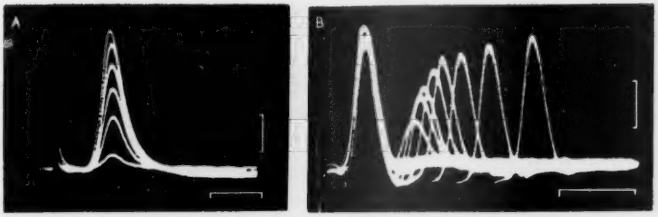

Fig. 12. The action potential of the olfactory nerve. A, superimposed records of responses to stimuli of increasing strength. Time mark 50 msec. Vertical bar 0.5 mV. B, the relative refractory period of the olfactory nerve. Time mark 100 msec. Vertical bar 200 μV.

is illustrated in Fig. 11. The response was reduced to half of its original height at a distance of about 1 mm from the bulb. The interesting point is, however, not the reduction in height but the appearance of a small potential that was uncovered as the bulb response decreased. This potential is seen in Fig. 11 B—D (upper row) as a negative wave ahead of p_1. The response is thus composed of three potentials: the initial negative wave, the bulb potentials p_1 and p_2. Repetitive stimulation makes the three components easily discernible (Fig. 11 A—B lower row) as it causes a depression and delay of p_2. The initial wave as well as p_1 are left unaffected. It was evident that the initial wave represented the synchronous volley entering the bulb. When the bulb was destroyed p_1 as well as p_2 disappeared (Fig. 11 C—D, lower row) whereas the initial wave was left behind unaltered.

The nerve action potential. The action potential of the olfactory nerve was in the following experiments recorded with Pt-electrodes and by using a condenser coupled amplifier with a time constant of 1 sec. The olfactory bulb was either pinched or removed in order to avoid interference from the bulb potentials. Fig. 12 shows the responses that were recorded when electrical shocks of increasing strength were applied to the mucosa. It may be noted that the increase in height of the action potential is not followed by any alteration in shape. The constancy of the response indicates a surprisingly high degree of functional uniformity of the olfactory fibres.

The excitability cycle of the olfactory nerve is illustrated by the record B in Fig. 12. The absolute refractory period had a duration of about 30 msec and was followed by a period of relative refractoriness lasting for about 200 msec. The existence of a supernormal period was examined in a few experiments that, however, gave no clearcut results.

The conduction velocity was measured in a number of experiments. Different sites in the nasal mucosa were stimulated with conduction distances from 2 to 6 mm. The average conduction velocity was found to be 0.14 m/sec (variation 0.11—0.22 m/sec).

Discussion

The investigation accounted for above was carried out as an extension of the previously reported studies on the electrical changes induced in the bulb by natural stimulation (OTTOSON 1954, 1959 a, b).

The results show that a synchronous volley entering the bulb sets up a response composed of two waves. The first wave, p_1, is a typical synaptic potential, whereas p_2 arises due to the propagated activity in secondary neurons. The differential nature of the two potentials is established by their behaviours under various experimental conditions. Thus, p_1 lacks a true refractory period and summates to a persisting negativity at stimulation frequencies above 20/sec. In striking contrast p_2 is not able to follow a tetanus higher than 1/sec owing to its long refractory period. While the origin of p_2 can safely be assumed to be in postsynaptic structures the site of production of p_1 is less clear. The constant latency of the response as well as the fact that antidromic activation did not affect p_1 speak in favour of a presynaptic origin. However, the failure of antidromic impulses to influence p_1 might be explained if it is assumed that the dendrites of the secondary neurons are not invaded.

As described by several anatomists (see *e. g.* CAJAL 1911) the glomeruli are large spherical bodies consisting of extremely thin nerve terminals that interlace with the dendrites of the mitral cells. A morphological feature of particular interest for the interpretation of the response is that there are no afferent endings on the mitral cell bodies. The synaptic contact between primary and secondary neurons is thus confined to the glomeruli. Any postsynaptic fraction of p_1 must therefore arise from activity of the mitral cell dendrites in the glomeruli. It was thought that test on occlusion, would give a definite clue as to the origin of p_1. The results are, however not conclusive since it cannot be excluded that the peripherally stimulated areas overlapped.

The present investigation has been confined to the analysis of the potential changes that can be recorded from the surface of the bulb. As mentioned above there is a distinct lamination of the nervous structures in the bulb. This structural organization appears to provide favourable conditions for a closer examination of the potentials with intrabulbar recordings from the various layers.

The first description of the electrical properties of olfactory fibres was given by GARTEN (1903) who recorded the action potential in the pike's olfactory nerve. The same preparation has recently been used by GASSER (1956) in electron microscopical studies on the fine structure of the olfactory nerves. In parallel electrophysiological experiments GASSER also investigated the functional properties of the nerves. The data obtained in the present investigation are in striking accordance with GASSER's observations. As pointed out by GASSER the simple configuration of the response implies a homogeneity in fibre size. In the pike the fibres have a mean diameter of about 0.2 μ and the variation in size is small.

The fundamental functional difference between nervous structures giving all-or-none responses and those producing graded responses has been established in numerous investigations (see *e. g.* KATZ 1950, GRAY and SATO 1953, EYZAGUIRRE and KUFFLER 1955). The classification into electrically excitable and inexcitable tissues has been emphasized by GRUNDFEST (1957). The experiment illustrated in Fig. 3 demonstrates that the olfactory receptors belong to the group of electrically inexcitable tissues. The receptor membrane thus exhibits similar properties as the postsynaptic membrane. If this similarity extends to a sensitivity of the receptor membrane towards substances that activate postsynaptic membranes remains to be investigated.

Summary

1. The potential changes induced in the olfactory bulb and nerve by electrical stimulation of the nasal mucosa have been studied in the frog.

2. When a single shock of 0.2 msec duration was applied to the olfactory epithelium the bulb developed a negative potential lasting about 150 msec and with an inflexion on the rising phase. The response was greatest in the rostral parts of the bulb and spread electrotonically out in the olfactory nerve. The caudal parts of the bulb yielded small positive potentials.

3. Repeated stimulation brought about a segregation of the response into two components. The first of these remained unaltered in amplitude during repeated stimulation whereas the second became smaller and was delayed at stimulation frequencies as low as 1/sec.

4. The first component lacked a true refractory period and summated to a sustained potential during repeated stimulation. The second component had an absolute refractory period of about 0.3 sec. This was followed by a relative refractory period lasting 2—3 sec.

5. An antidromic volley backfired into the bulb blocked the second component but left the first component unaffected.

6. Asphyxia or narcotics depressed the response. The second component was most vulnerable and was the first to be abolished.

7. If two volleys were sent into the bulb simultaneously from different parts of the sensory epithelium the responses did not sum in an additive manner. Both components occluded.

8. It is concluded that the first component is a synaptic potential arising in the glomeruli. The second component is ascribed to the propagated activity in secondary olfactory neurons.

9. Observations are presented which are regarded as evidence that the olfactory end organs are electrically inexcitable. When electrical shocks are applied to the nasal mucosa the afferent fibres appear to be directly excited.

10. In recordings from the olfactory nerve the electrotonically conducted

bulb response was preceded by the propagated spike. This was obtained in isolation after the bulb had been pinched.

11. The most characteristic feature of the olfactory nerve action potential was its simple configuration. The conduction velocity was about 0.14 m/sec, the absolute refractory period 30 msec and the relative refractory period about 200 msec.

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Metastatic Calcification in Experimental Overdosage of Dihydrotachysterol in Rats

Effect of Tetracycline, DOCA and Cortisone

By

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Abstract

HÄKKINEN, I. P. T. Metastatic calcification in experimental overdosage of dihydrotachysterol in rats. Effect of tetracycline, DOCA and cortisone. *Acta physiol. scand.* 1959. *47.* 173—178. — Tetracycline and DOCA were observed to increase metastatic calcification in rats treated with dihydrotachysterol (AT_{10}). Cortisone had an opposite effect. DOCA increased tetracycline fluorescence in calcium metastatic regions, cortisone had again an opposite effect. So tetracycline fluorescense and metastatic calcification seemed to correlate. The way of fixation of tetracycline has been discussed.

Observations that Toluidin Blue increases metastatic calcification in overdosage of parathyreoid hormone in rats have been made (BAKER, REAVEN and SAWYER 1953, 1954). As the widely discussed ground substance (GERSH and CATCHPOLE 1949, SHETLAR *et al.* 1956, and others) depolymerizes under the effect of parathyreoid hormone, BAKER *et al.* (1954) assume that Toluidin Blue combines with chondroitin sulphate so that the ability of combining calcium also increases. On the other hand MILLER, WALDMAN and MC LEAN (1952)

Table I. Metastatic calcification in rats after AT_{10}. Tetracycline fluorescence in metastatic areas.

No. of animals	Treatment	The grade of fluorescence					
		Kidney					
		0		+		++	
		no.	%	no.	%	no.	%
7	AT_{10}+tetracycline	0	0	2	29	5	71
12	AT_{10}+tetracycline+cortisone	5	42	5	42	2	16
14	AT_{10}+tetracycline+DOCA	1	7	7	50	6	43
5	Tetracycline	5	100	0	0	0	0

have succeeded in preventing ossification of rachitic cartilage in rats with Toluidin Blue. BAKER *et al.* (1954) are not able to explain what causes this in a way opposite effect. BAKER *et al.* (1954) also studied the effect of DOCA on hyperparathyreoidism in rats and observed diminished calcification in the kidneys in 5 animals. This was explained by the phlogistic effect of DOCA, which would be the same as the increasing of polymerisation degree in ground substance. This conclusion sounds peculiar, as we know that mucopolysaccharides depolymerize in active connective tissue (GERSH *et al.* 1949). They further assumed that cortisone increases metastatic calcification. In some later works BACON, PATRICK and HOWSARD (1956) and LARON, MUHLETHALER and KLEIN (1958) observed that cortisone prevents calcification of kidneys in experimental hyperparathyreoidism. These investigations have mainly been undertaken to explain the function of parathyreoid hormone, but at the same time light has been thrown on the phlogistic-antiphlogistic effect of DOCA and cortisone, which (TURNER 1955, p. 197) seems evident. Some important qualities of the connective tissue have been examined in experimental conditions and it has been found that all three hormones, parathyreoid, DOCA and cortisone have an effect on it.

A further observation connected with the reaction of connective tissue (HÄKKINEN 1958) was the fixation of tetracycline in calcium metastatic regions produced by AT_{10} in rats. The further development of this observation led to the present work, where the aim is to explain the relation of fluorescing tetracycline in connective tissue in reactive state, and especially the effect of cortisone-DOCA on it.

Materials and Methods

Male albino rats, age about 7 months and mean weight 200 g, were employed as experimental animals. During the experiment the animals were given ordinary laboratory food and water. The animals were divided into following groups:

Effect of cortisone and DOCA on the fluorescence. See text.

The grade of fluorescence												
Stomach						Heart muscle					
0		+		++		0		+		++	
no.	%	no.	%	no.	%	no.	%	no.	%	no.	%
1	14	0	0	6	86	3	43	1	14	3	43
4	33	6	50	2	17	7	59	4	33	1	8
0	0	7	50	7	50	4	28	4	28	6	44
5	100	0	0	0	0	5	100	0	0	0	0

Group 1: 7 animals. These were given 10 mg of AT_{10} through an oral tube and 5 mg on the following day. The rats received a single injection of tetracycline i. m., 50 mg/kg body weight.

Group 2: 12 animals. These were given AT_{10} and tetracycline in the same way as group 1 and also 5 mg of cortisone acetate/day s. c.

Group 3: 14 animals. These were given AT_{10} and tetracycline in the same way as group 1, besides 1.5 mg DOCA/day i. m.

Group 4: 5 animals. AT_{10} in the same way as group 1.

Group 5: 5 animals. Tetracycline in the same way as group 1.

The injections of DOCA and cortisone were begun 2 days before feeding AT_{10} and they were continued for 7 days. AT_{10} was given in all 15 mg on 2 days and a single dose of tetracycline (Lederle Laboratory kindly presented the "Achromycin intravenous" used in the experiment), 4 days before the sacrification the dose being 50 mg/kg body weight. The rats were sacrified on the 8th day. The bones, kidneys, stomach and heart muscle were examined in UV-light. Histological specimens were taken from the yellow fluorescing regions of these organs. If there was found no fluorescence, a specimen was taken in the same way as from the fluorescing tissues. The specimens were fixed in formalin and stained with hematoxylin and eosin.

Results

In all other groups except that having received DOCA a decrease in weight was noted. The animals of the DOCA group either maintained their former weight or the weight increased slightly. This phenomenon may be explained by the retention of electrolytes and water. The weight in the group which received only tetracycline remained unaltered. The fluorescence caused by tetracycline was examined as previously reported (HÄKKINEN 1958). In corresponding histological specimens dark blue spots caused by calcium were examined. These contained necrosic parts and inflammatory cells in the heart muscle and in the connective tissue of the stomach. Dark blue spots were noted in the basal membrane of the cells in the kidneys and dark blue masses in the lumen of the tubulus was also found in more calcified tissues. BAKER *et al.*

Table II. Metastatic calcification in rats after AT_{10}. Effect of tetracycline, DOCA and cortisone

No of animals	Treatment	The grade of calcification					
		Kidney					
		0		+		++	
		no.	%	no.	%	no.	%
7	AT_{10}+tetracycline	1	14	6	86	0	0
12	AT_{10}+tetracycline+cortisone	3	25	9	75	0	0
14	AT_{10}+tetracycline+DOCA	1	7	10	71	3	22
5	AT_{10}	4	80	1	20	0	0
5	Tetracycline	5	100	0	0	0	0

(1954) have divided the calcified tissues into groups on the ground of this staining. The following grouping has been used in the tables: a negative result, slightly positive and strongly positive result. In Table I the different organs have been classified in reference to the fluorescence caused by tetracycline. 0 = no fluorescence, + = weak or only regional fluorescence, ++ = strong fluorescence.

The histological results have been classified in Table II. 0 = no dark blue colour caused by calcium. + = only in few places appearing colour. ++ = a strong dark blue colour in several places.

The difference between weak and strong fluorescence is not only the difference in intensity. The weak fluorescence means spotted slight yellow colour. The strong fluorescence is a compact colour.

The results show, that cortisone decreases the fluorescence of tetracycline and also calcification. The effect of DOCA is opposite and clear: fluorescence increases as well as calcification.

Comparing the group having received only AT_{10} with the group AT_{10}+ tetracycline there is noted more calcification in the latter than in the former. Comparing Table I and II in regard to the groups having received tetracycline it is found that the histological picture and tetracycline fluorescence correspond to each other rather well. The conclusion may be drawn that tetracycline fixes approximately in the same places as calcium and at the same time enhances calcification.

Discussion

On the basis of the works by RUBIN and HOWARD (1950), ENGEL (1952) BAKER *et al.* (1953, 1954) and SHETLAR *et al.* (1956) it may be considered evident that the parathyreoid gland has a direct effect on connective tissue and causes changes in the mucopolysaccharides of the ground substance. LASKIN and

on the calcification. See text.

The grade of calcification											
Stomach						Heart muscle					
0		+		++		0		+		++	
no.	%	no.	%	no.	%	no.	%	no.	%	no.	%
3	43	4	57	0	0	2	29	3	42	2	29
5	42	7	58	0	0	7	58	4	34	1	8
1	7	11	79	2	14	1	7	7	50	6	43
4	80	0	0	1	20	0	0	4	80	1	20
5	100	0	0	0	0	5	100	0	0	0	0

ENGEL (1956) have studied the biochemistry of the bones more thoroughly in this connection and observed that the succinic dehydrogenase activity diminishes in overdosage of parathyreoid hormone. The oxygen consumption diminishes, glycogen decreases and the mucoproteins of the ground substance disaggregate. These effects are evidently on an intracellular level.

The mechanism has not been explained how calcification on the changed ground substance of soft tissues takes place. A corresponding calcification occurs in connection with the vitamin D and dihydrotachysterol (AT_{10}) overdosage (CARLSSON and LINDQUIST 1955, CRAWFORD *et al.* 1957), which has also been used in the present work.

Toluidin blue has before been used in experiments as a factor increasing metastatic calcification, (BAKER *et al.* 1953, 1954). SELYE (1957) has also used Phenylbutazone in the same way. The result of BAKER *et al.* in regard to DOCA is perhaps not right and they do not consider it reliable even themselves, because of the small material.

In the present work DOCA and tetracycline were observed to be factors increasing metastatic calcification in the overdosage of AT_{10}. Cortisone was observed to be a factor decreasing calcification. Because according to BAKER *et al.* (1954) Toluidin blue combines with chondroitin sulphate it would be tempting to think that tetracycline behaves in the same way. This assumption is perhaps supported by the observations of the present writer that after the hydrolysis the content of sulphate is greater in the tissues fluorescing with tetracycline than in their neighbouring healthy tissues (HÄKKINEN et HARTIALA 1959 and in preparation). These analyses have been carried out with experimental cincophen ulcers of dogs.

Heparin and Toluidin blue are antagonists in blood coagulation mechanism (ALLEN *et al.* 1949). It is interesting to see the relation of tetracycline and heparine to each others. It is perhaps appropriate to see the effect of tetracycline on ossified cartilage.

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The Kinetic Energy Produced by Voluntarily Controlled Muscle Action and the Frequency of the Motor Discharge

By

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Abstract

BERGSTRÖM, R. M. The kinetic energy produced by voluntarily controlled muscle action and the frequency of the motor discharge. *Acta physiol. scand.* 1959. *47.* 179—190. — The experiments were performed in order to study the voluntary control of muscle contraction. Under the experimental conditions concerned it was possible to demonstrate a linear relationship between the discharge frequency of the electromyogram recorded from the forefinger abductor muscle using non-selective electrode technique and the kinetic energy produced by the abduction. It is assumed that the mechanical effect of voluntary muscle contractions is graded by the central nervous system by the gradation of the number of the time periods in the motor discharge. The number of the time periods is proportional to the physical action (energy · time, dimension $g \cdot cm^2 \cdot s^{-1}$) of the mechanical effect of the motion.

As is well known the central nervous system directs the functioning of a muscle in connection with voluntary actions by varying not only the frequency and duration of the motor discharge, but also the number of the active motoneurones (ADRIAN and BRONK 1929, BRONK and FERGUSON 1935, GILLSON and MILLS 1941, GESELL and ATKINSON 1943). Though these factors are,

according to what is called Adrian-Bronk's law, a kind of measure of the nervous activity, it is still obscure what exactly is their interrelationship and what part each of them plays in the mechanical performance of a muscle. In studies designed to elucidate the correlation between the electrical activity of the motor nervous system and the mechanical activity of a striated muscle the point of departure has mostly been the motor unit. One has nevertheless been unable to find, for one single anatomico-physiological unit of such a kind, any simple relations in the sense concerned here, which is partly attributable to the fact that, as pointed out by GELLHORN (1953), different neurones show widely different characteristics in threshold, frequency of discharge and rate of adaptation. Since, however, man is capable of even exceedingly accurate motor performances in his voluntary actions, it would be desirable to demonstrate the mode of this regulating mechanism within the control of muscle functions exercised by the central nervous system.

The fact that it is actually the motions of his body and limbs relative to his environment, rather than the functions of individual motor units or muscles, that man directs in moving (HORSLEY 1909, WALCHE 1943), gives rise to the assumption that the regulation of the electrical activity of the central nervous system does not pertain to the functions of the anatomico-physiological structures of a muscle, but to a motor performance with respect to a certain environment. Such an assumption is suggested, for example, by injuries of the cerebral cortex whereby appropriate motor performances are impaired without there being any disorder in the regulation of the activity of the motor units or muscles concerned. Sense physiological investigations also show that the "subjective", voluntary control (see REENPÄÄ 1959, BERGSTRÖM 1957, 1958 a) of muscle activity is concerned with the physical relationships of the motor performance within the environment, such as the distance travelled by a limb (JALAVISTO *et al.* 1937) or the mechanical effect of the limb on its environment (BERGSTRÖM 1957, 1958 a).

It may accordingly be assumed that it is possible to find a simple relation between the mechanical effect of a muscle and the electrical activity of the motor nervous system controlling it, by investigating the dependence of the mechanical effect caused by the muscle performance in the environment upon the discharge of the motoneurone group "innervating the motion". In the most simple case where the motion is accomplished using one single small muscle alone this interdependence can be studied by means of the non-selective electrode technique, the electromyogram yielded by it giving a picture of the electric activity appearing in the whole muscle (JALAVISTO *et al.* 1938, BERGSTRÖM 1959) and the corresponding motoneurone group. This procedure is based on the justifiable assumption that in the electromyogram the addition and subtraction between the potentials of the motor units, arising in different parts of the muscle, remain constant when the activity of the muscle is increased (BIGLAND and LIPPOLD 1954). On the other hand, from an electro-

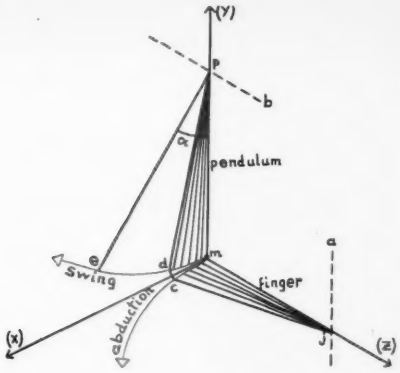

Fig. 1. Schematical representation of the method used in recording the kinetic energy produced by the abduction of the forefinger (see text).

myogram recorded by means of non-selective surface electrodes it is possible to count the "spikes" of the muscle action currents up to a frequency of appr. 500 cps (BERGSTRÖM 1959). It can thus be supposed, as far as small muscles are in question, that the frequency recorded from surface electrodes can be considered as a "sample" representing the frequency in the whole muscle. It may be suitable for use, in particular, in large materials, where the random differences between the samples cancel out in the statistical treatment.

As, in unselected subject materials, the voluntary control of muscle activity is concerned, according to some sense physiological studies (BERGSTRÖM 1957), with the kinetic energy brought about by the action within the environment, it can be assumed that the electric activity of the motor nervous system bears a linear relation to this physical magnitude. On the other hand we know, as indicated above, that according to Adrian-Bronk's law the frequency of discharge of an individual motoneurone and the number of active motoneurones are, aside from the duration of the motor discharge, the factors through which the central nervous system regulates the muscle activity (BERGSTRÖM 1958 a, b). The frequency, however, which is obtained as total output with non-selective electrodes from a muscle entails the recruitment of motoneurones. Thus it is possible to assume that the magnitude descriptive of nervous activity which determines the amount of kinetic energy within the environment, brought about by the motion, is the total frequency for the muscle. This being so, the problem of the present study can be posed in the following form

$$E = H_e \cdot \nu_\tau, \tag{1}$$

where E stands for the energy produced by the muscles in the environment, ν_τ for the frequency of the discharge of the motoneurone group (a muscle or group of muscles) innervating the motion and H_e is a coefficient of proportionality.

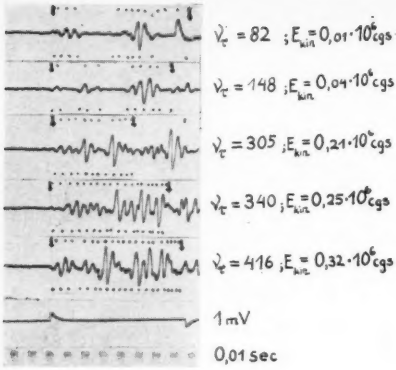

Fig. 2. Electromyogram recordings obtained with surface electrodes from m. interosseus dors. I by abductions of the forefinger. The arrows indicate the duration of the touch between finger and pendulum. The dots indicate the estimation of the frequency of the motor discharge. The mean frequencies (ν_t) during 0.01 sec preceding detachment (second arrow), and corresponding kinetic energies (E_{kin}), on the right.

Methods

The experiments were carried out with the abductor muscle of the right forefinger, which has been the subject of sense as well as muscle physiological investigations in our studies referred to above. The experimental set-up, represented schematically in Fig. 1, was the same as in some of our previous papers (BERGSTRÖM 1958 a, b). The experimental subjects were required to perform abductions of the forefinger, the kinetic energies of which were transmitted to a pendulum and could be determined, provided that the mass and the amplitude of the swings of the pendulum were known. In Fig. 1 *pm* is the length of the pendulum swinging, due to the strokes, in the vertical plane *x,y* about the axis *pb* (*mde* being the swing described by the mass *m* of the pendulum) and α denotes the angle of swing. The segment *jm* represents the forefinger which is fixed to a light and easily moving rail and which is abducted in stroke, in the horizontal plane *x,z*, about the axis *ja* through the proximal joint (*j*). Prior to the experiment the forefinger was placed so as to touch, at the distal joint, the mass *m* of the pendulum then at rest. In the figure the shaded areas (*pmd* and *jmc*) represent the distribution of contact in the stroke, *cd* indicating where the finger touches the mass. The distance *jm*, *i. e.* that of the point of contact from the proximal joint, was the same in all the experiments and so was the length *pm* of the pendulum. At the beginning of the stroke the finger was at its rest position on the rail, the palm being supported by a stable plaster mould. The experiments could not be arranged so as to avoid a small shift *cd* of the point of contact between the finger and the mass of the pendulum and a consequent energy loss in transmitting the mechanical effect from the finger to the pendulum, the magnitude of this loss being dependent on that of the shift. This energy loss, however, was estimated to be small relative to the energy transmitted to the pendulum.

The weight of the mass *m* was varied in the experiments using additional weights ranging from 0 to 150 g. In doing so consideration was given to the shift of the centre of gravity due to the additional masses and the consequent effect upon the length *pm* of the pendulum. The physical characteristics of the pendulum, devised especially for the purpose of the experiments of the present type, and the method of determining the kinetic energy produced by the stroke on the basis of the mass *m* and the angle α have been described in connection with an earlier work (BERGSTRÖM 1957). Determination of the angle of oscillation took place by direct visual observation. The accuracy of the observer was controlled by special experiments.

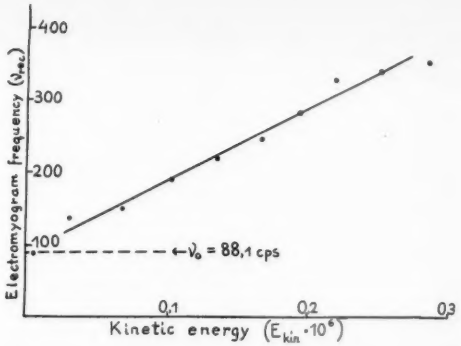

Fig. 3. Kinetic energy and frequency of the motor discharge in forefinger abduction.

During the stroke the action currents of the abductor (m. interosseus dors. I) of the forefinger were recorded in the usual manner from surface electrodes, an asymmetric amplifier and a cathod-ray oscillograph on a film travelling at 50 cm/sec. The electrodes were situated on the muscle to be investigated (Ø 0.3 cm) and in the distal-dorsal part of the forearm (Ø 0.6 cm) just as in the study dealing with the counting of impulses in an electromyogram which was referred to above (BERGSTRÖM 1959). To record the duration of the contact the finger and pendulum and to mark it on the myogram use was made of an electron relay (Philips). During the experiments we took pains to keep the experimental conditions and, in particular, the location of the electrodes constant. To avoid fatigue short series of experiments were used.

The kinetic energy (E_{kin}) of a body is $E_{kin} = m \cdot v^2/2$ (m = mass, v = velocity). As in our experiments the velocity affecting the energy that the pendulum receives in the stroke depends on the state of motion in which the finger is at the moment it ceases to touch the pendulum (Fig. 1, the instant of time corresponding to point d in the plane of swing x,y), the potential frequency of the muscle ought to be determined for this very instant. However, the time interval between the action potentials of the different motor units, *i. e.* the time period τ defining a certain frequency $\nu_\tau = n_\tau/t$ (where n_τ is the number of time periodes τ in the time t), is subject to too large random variations for being used in the determination of the frequency prevailing in the muscle at the moment of detachment. The frequency was determined as the mean of the time periods τ contained in the time 0.01 sec immediately preceding the moment of detachment. The principle adopted in estimating this frequency is evident from Fig. 2. The electrocardiograms shown in the figure represent strokes whereby the kinetic energy received by the pendulum was between 0.01 and $0.32 \cdot 10^6$ cgs units. As has been pointed out previously (BERGSTRÖM 1959), we found it appropriate to consider every "spike" of the electromyogram as an action potential irrespective of its location and size.

Experiments were carried out with 10 subjects, the total number of recordings being 400. All of the subjects had had practice in using the muscle concerned in previous studies, which was considered important in view of the fact that the directed motions of the abductor of the forefinger are usually poorly developed as it is not ordinarily used in giving strokes.

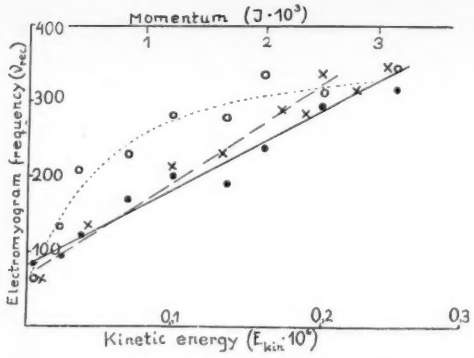

Fig. 4. A typical (full circles) and a divergent (hollow circles) series of the same subject on different days. Crosses and broken line: momentum (I) of the divergent series plotted against frequency (see text).

Results

Fig. 3 is a graphical representation of the results obtained in all of the experiments performed. In the figure the frequency of the motor discharge, estimated from a myogram (such as represented in Fig. 2), at the moment when the mass of the pendulum leaves the finger giving the stroke is plotted against the kinetic energy, in cgs units, obtained by the pendulum in the stroke. Each of the plotted points is the mean of 40 observations, the standard deviations varying between $\pm$ 8.5 and $\pm$ 21.6 cps.

The relation between the electrical and mechanical activity is linear, though the dispersions are large. As indicated above, however, this could be expected. The largeness of dispersions is also attributable to the changes in the elasticity conditions of the finger in the stroke, which are dependent on the different kinds of masses. It can be seen from the figure that the line obtained intersects the vertical axis at $\nu_{rec} = 88.1$ which point (ν_o) has been indicated by an arrow. It is necessary for the muscle to have this frequency in order that the system made up of the finger and the pendulum be set in motion. Consequently, there is a certain value E_o of energy which is necessary for overcoming the resistance of the system. This resistance is mainly the result of the inertial forces within the finger and the muscle (resistance in the axis of the pendulum and the rail being small), so that the energy E_o corresponding to the frequency ν_o is a kind of a value of static energy, necessary for bringing about the motion of the limb in its environment.

Fig. 4 shows two experimental series made with the same subject on different days. The first of them (full circles) represents a typical and the second one (hollow circles) a divergent result. Each of the full and the hollow circles

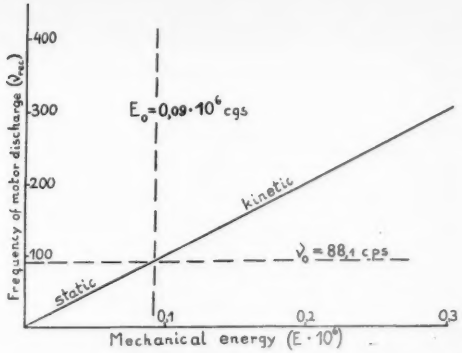

Fig. 5. Distribution of the mechanical energy (E) produced by forefinger abduction. E_o = static energy necessary for initiating the motion; E_{kin} = kinetic energy dependent on the state of motion. ν_o = motor frequency of the abductor muscle necessary for E_o.

plotted depicts only one single observation. The divergent series can be considered to result from intraindividual variations in the control of voluntary muscle contraction (BERGSTRÖM 1957), for the results of the same individual on the other days, including the series represented by the full circles, were typical. If the divergent series is represented plotting the momentum $I = m \cdot v$ which the pendulum receives in impact (scale in the upper edge of the figure) against frequency, the resulting curve is a straight line (broken line, crosses).

Fig. 5 is a graphical representation of the distribution of the energy E, corresponding to the potential frequency recorded from the abductor of the forefinger, between the static part E_o, which brings about the motion of the finger, and the kinetic part E_{kin}, which is dependent on the state of motion of the finger. The value E_o is obtained assuming that frequency bears the same relation to energy in the static as in the kinetic phase of the process. For the static energy we get $E_o = 0.09 \cdot 10^6$ cgs units, corresponding to a frequency of the motor discharge of $\nu_{rec} = 88.1$ cps (Fig. 3).

Taking account of the assumption underlying equation (1) the typical experimental results may be represented as follows

$$E = E_o + E_{kin} = a \cdot H_e \cdot \nu_t \quad (H_e = \text{const.}), \tag{2}$$

where E stands for the total energy ($E = E_o + E_{kin}$) generated in muscle contraction and ν_t for the frequency of the discharge of the motoneurone group innervating the motion, in the present case the total frequency of the abductor of the forefinger. H_e is a proportionality coefficient, which is constant according to the experimental results, a being a coefficient equal to the ratio of the potential frequency ν_{rec} recorded from the electromyogram to the potential frequency ν_t actually appearing in the muscle, *i. e.* $a = \nu_{rec}/\nu_t$. If the

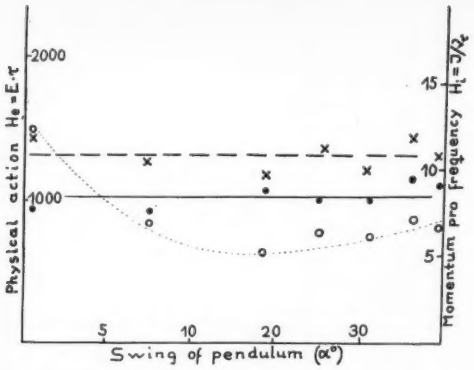

Fig. 6. The physical action (energy · time; $H_e = E \cdot \tau$) corresponding to one time period (τ) and the strength of the stroke measured as angle ($\alpha°$) of swing of the pendulum. Full circles and continuous line: a typical series, hollow circles and dotted line: the divergent series, crosses and broken line: the divergent series corresponding to the vertical scale H_i, see text.

recorded value is the same as the true value of the potential frequency of the muscle, the value of a is unity.

H_e, *i. e.* the coefficient representing the relation between the mechanical energy of the muscle and its electrical frequency, has the dimensions of physical "action" (energy · time, in German: Wirkung), $g \cdot cm^2 \cdot s^{-1}$, so that it may be regarded as an expression for the "action" that is generated in the process:

$$H_e = (1/a) \cdot E/\nu_T = (1/a)\ E \cdot \tau = \text{const.}\ (g \cdot cm^2 \cdot s^{-1}) \tag{3}$$

In Fig. 6, the experimentally determined values of H_e, *i. e.* the action corresponding to one time period, have been plotted against those of the strength of the stroke, the latter being measured from the angle of swing α of the pendulum. As is evident from the figure, which represents a typical series (full circles), the action $H_e = E \cdot \tau$ that corresponds to one time period remains constant despite the variations in the strength of the stroke (the numerical value of H_e being under the experimental conditions concerned = 970 cgs units). The divergent series of results shown in Fig. 4 has also been plotted in this figure (hollow circles). In the divergent series the action (H_e) decreases with increasing strength of the strokes (amplitude of the swings). Replacing the energy E in equations (2) and (3) by the momentum I which the bob of the pendulum receives in the stroke and writing H_i for the ratio between this momentum and the electrical frequency of the muscle we get, for the divergent series, the result represented by the crosses and broken line in Fig. 6. The vertical H_i-scale for the crosses and broken line is on the right. H_i remains constant when the strength of the strokes is varied.

Discussion

According to the results presented above the kinetic energy produced by the voluntary abduction of the forefinger bears, in the typical case and under the experimental conditions concerned, a linear relation to the impulse frequency of the electromyogram with surface electrodes from the muscle (m. interosseus dors. I). The experimental result (Fig. 3 and 5) was written formally as

$$E = E_o + E_{kin} = a \cdot H_e \cdot \nu_I, \text{ where} \tag{2}$$

$$H_e = (1/a)\ E/\nu_I = (1/a)\ E \cdot \tau = \text{const.}\ (g \cdot cm^2 \cdot s^{-1}). \tag{3}$$

Here E stands for the total mechanical energy produced by the muscle contraction, E_o for the static energy required to bring about the motion of the finger and to overcome the resistance of the finger tissue and muscle, and E_{kin} for the kinetic energy that depends on the state of motion of the finger. ν_I is the frequency of the discharge of the motoneurone group innervating the motion. The coefficient a represents the ratio between recorded frequency and the true frequency appearing in the muscle, so that $a \cdot \nu_I$ is the result obtained from the myogram. H_e is a proportionality coefficient representing the relation between the energy and the frequency of discharge. From equation (2), H_e has the dimension $g \cdot cm^2 \cdot s^{-1}$ and is accordingly descriptive of the physical "action" (energy · time) $E \cdot \tau$, where τ is the time period defined by the frequency ν_I. According to the results obtained the magnitude H_e remains constant in muscle contraction in spite of the variations in the strength of the stroke (Fig. 6).

As demonstrated previously (BERGSTRÖM 1958 a, b), the nervous factors participating in the regulation of the mechanical activity of a muscle, *i. e.* the number of active motoneurones (recruitment) and the frequency and duration of their activity, may be represented in the form

$$n_I = \nu_I \cdot t, \text{ when } \nu_I = 1/\tau, \tag{4}$$

where n_I is the number of motor impulses or, more accurately, the number of time periods τ, discharged into the muscle through the motoneurones, t being the time during which these n_I impulses reach the muscle. ν_I is the joint frequency of the motoneurones active during the motion.

If the frequency recorded from a myogram is considered the true frequency, *i. e.* if a in (2) is taken to be unity, we have

$$E = H_e \cdot \nu_I \tag{5}$$

and since, on account of (4), $\nu_I = n_I/t$, we also have $E = (n_I \cdot H_e)/t$ or

$$H_t = \int E \cdot dt = n_I \cdot H_e\ (g \cdot cm^2 \cdot s^{-1}). \tag{6}$$

In (6), H_t has been written for the physical action (energy · time) transmitted, within the musculature, to the limb and the environment during the time t, which is the duration of the muscle contraction.

The result (5) indicates that the central nervous system regulates the kinetic energy transmitted to the environment in connection with voluntary muscle contractions by gradating the joint frequency of the motoneurones active during the motion.

As our experiments indicated H_e to be constant and as $n_t = \nu_t \cdot t$ in virtue of (4), equation (6) shows that the central nervous system is capable of gradating the mechanical effect of the muscle, *i. e.* the action $H_t = \int E \cdot dt$, by firing a given number of impulses to the muscle. Thereby it is immaterial, from the point of view of the mechanical action H_t, during which time or at which frequency the impulses reach the muscle, for the action is then only dependent on n_τ. The number of the time periods (number of motor impulses) can consequently be regarded as the essential central factor in the gradation of the mechanical muscle activity in a typical voluntary contraction.

As is evident from (6), a prerequisite for such a regulation is, however, the constancy of the action $H_e = E \cdot \tau$. All the factors that affect the relationship between the mechanical effect and the electric activity in a muscle, such as fatigue (LOOFBOURROW 1948, SHERRER, SAMSON and SOULA 1954), temperature (JALAVISTO, KERÄNEN and SEPPÄLÄ 1939), stimulation from the adrenosympathetic system (BROWN, BÜLBRING and BURNS 1948), proprioceptive reflexes (GELLHORN 1953, HOLMGREN and MERTON 1954), individual variations (BERGSTRÖM 1957) etc., are naturally capable of giving rise to variations in H_e, with the result that the relationship (6) breaks down in a way similar to that exemplified by the divergent series plotted in Fig. 4 and 6.

It may nevertheless be assumed that the voluntary control of muscle functions, *i. e.* the gradation of the mechanical effect of a muscle, is founded on the constancy of H_e, which constancy can be "relied upon" by the central nervous system in gradating the activity of muscles (perhaps by means of the physiological mechanism controlling muscle spindle activity, according to GRANIT and KAADA 1953).

The results incorporated in equations (5) and (6) are not surprising in themselves, providing that account is taken of the fact that what we are concerned with in voluntary muscle contraction is the interdependence of two processes, one of which is a mechanical motion phenomenon, the other one being a periodical process.

It can thus be seen that a given action $H_e = E \cdot \tau$ is associated with each time period of the discharge of the motor nervous system, determining the energetic value of the time period, when it reaches the muscle and acts upon the environment. Therefore, and as the periodical process of a motor nerve is the primary excitement to voluntary muscle contraction, it may be advantageous in practice to define the "quantity" of the nervous motor time period in terms of the accompanying action (H_e) according to equation (6).

This is tantamount to saying that the process underlying the motor spike (time period) is a kind of "quantum", whose frequency determines the amount

of energy transmitted from the muscle into the environment. This "quantum" has, according to the formal expression given by equation (6), the dimensions of physical action ($g \cdot cm^2 \cdot s^{-1}$).

It should be remembered, however, that our empirical equations are only statistical expressions of some elementary processes in the muscle cells, which are only valid for a great number of these elementary processes. In order to study the problems involved in the interaction between the periodical and the mechanical phenomenon in the muscle, we should not pay regard only to the anatomico-physiological system of the motor units, but rather to the statistical relations between different forms of processes as a whole.

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The Mechanical Work Produced by Voluntarily Controlled Muscle Action and the Frequency of the Motor Discharge

By

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Abstract

BERGSTRÖM, R. M. The mechanical work produced by voluntarily controlled muscle action and the frequency of the motor discharge. *Acta physiol. scand.* 1959. *47.* 191—198. — The interrelationship between the mechanical work and the impulse frequency of the electromyogram obtained with non-selective electrodes was studied in voluntary actions of the human forefinger abductor muscle. In view of the fact that the work performed by the muscle is independent of the time relations of the contraction, the impulse frequency corresponding to a certain amount of mechanical work was registered in the terminal position of the muscle shortening. The impulse frequency of the electromyogram was directly proportional to the degree of the muscle shortening by different loadings of the contractions and to the amount of work produced by the unloaded contraction. The result makes it possible to treat the impulse frequency of the motor discharge as an energy parameter, which shows the amount of the "potential energy" corresponding to a certain length of the muscle.

When a muscle contracts in voluntary action under a certain load and at the same time shortens, a certain amount of work is done by the force that is active in the muscle and moves the limb. The work done by the muscle is dependent on the magnitude of the force and the distance travelled by its

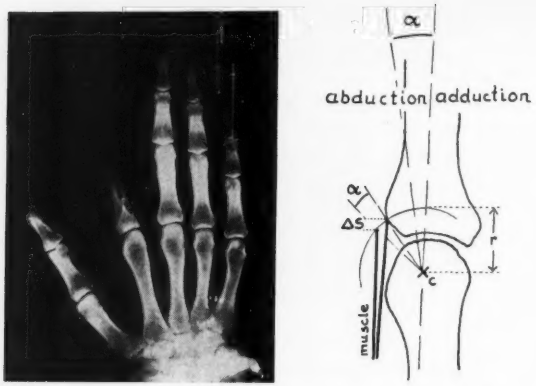

Fig. 1. The proportionality of the shortening of m. interosseus dors. I, $\triangle s$, to the amplitude of the abduction movement of the forefinger, $\alpha \cdot r$ = distance of insertion point from the axis of movement. A schematical representation drawn on the basis of roentgenograms.

point of application in the direction of the force, *i. e.* on the muscle shortening, according to equation $A = k \cdot \triangle s$, where A stands for the work done in the action, k for the force developed by the muscle and $\triangle s$ for the shortening of the muscle.

When a muscle shortens in connection with a voluntary, isotonic contraction, the increase in the muscle shortening is accompanied by an increase in the impulse frequency of the electromyogram (JALAVISTO *et al.* 1938). Then there is an increase in the impulse frequency of both the whole muscle and the motor unit. The study referred to does not give any more detailed information on the nature of this inter-relation. Since, as we have shown before, the kinetic energy produced by a muscle in voluntary action increases in direct proportion to the impulse frequency of the electromyogram (BERGSTRÖM 1959 b), it is interesting to examine in greater detail the interdependence of the shortening of the muscle, affecting the work done by it, and the impulse frequency. This is the objective of the study reported in the following.

Method

The experiments were carried out with the abductor muscle of the forefinger (m. interosseus dors. I) as in the study of JALAVISTO *et al.*, referred to above. The right hand of the subject was fixed in a plaster mould, his forefinger being stretched out and supported by an easily moving, light rail, whose axis of movement coincided with the abduction axis of the finger. The abduction took place in the horizontal plane. The abductor muscle was loaded using hanging weights so that a force of the desired magnitude was transmitted to the finger, (to its distal phalanx 7 cm from the abduction axis) through a torsion-free cord and a wheel with bearings. To measure the shortening

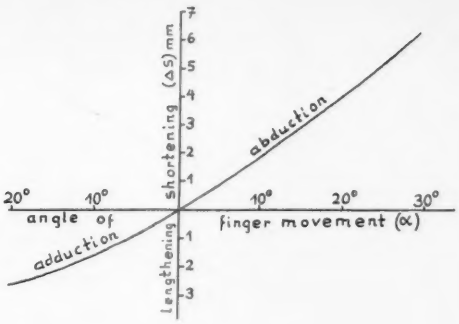

Fig. 2. Abduction and adduction angle of the forefinger, α, and the shortening of m. interosseus dors. I, $\triangle s$.

of the muscle X-ray photographs were taken of the hands of the subjects at different degrees of abduction. As is evident from Fig. 1, there is a change in the proportionality of the shortening of the abduction muscle ($\triangle s$) and the abduction angle (α) of the finger when the abduction is increased. In the experiments, where the angle of abduction was varied between 0 and 30°, the shortening of the muscle varied between 0 and 0.63 cm in a way depicted in Fig. 2.

The loading of the abductor muscle was done by using weights of different magnitudes so that the force acting upon the distal insertion point of the muscle and resisting the abduction varied between $1.7 \cdot 10^5$ and $6.8 \cdot 10^5$ dyne. To determine this force the motion axis of the finger and the moment relations were determined empirically from the X-ray pictures for each subject.

The muscle action currents were recorded, on a running film (50 cm/s), during the loading at the different degrees of abduction in the customary manner by surface electrodes using an amplifier and oscillograph. The size of electrodes (∅ 0.3 cm on the muscle and 0.6 cm on the distal-dorsal skin of the forearm), the degree of amplification and the other recording conditions were kept constant during the experiments. As for the determination of the impulse frequency from the electromyogram we refer to our earlier studies (BERGSTRÖM 1959 a, b). Experiments were carried out with three subjects so as to obtain 30 serviceable electromyogram recordings for each loading and degree of abduction. To avoid fatigue phenomena the experimental series were short in each sitting. Following each recording there was also a pause. After the pause the finger was again placed passively into the abduction degree, which enabled us to eliminate the effect of the speed of shortening of the muscle upon the results of registration.

Results

Fig. 3 shows the dependence of the impulse frequency of the electromyogram, v_i, upon the shortening of the muscle, $\triangle s$, for different loadings (white and black circles and squares, continuous lines). The points represent the means of 30 recordings, the standard deviation varying between $\pm$ 7.4 and $\pm$ 19.6 cps. For each curve the magnitude of the force, in dyne, acting at the distal insertion point of the muscle has been indicated.

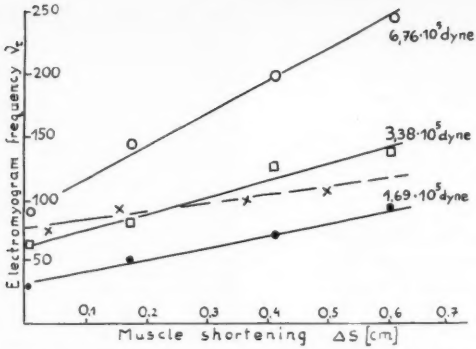

Fig. 3. Interdependence of the shortening of m. interosseus dors. I ($\triangle s$) and the impulse frequency (ν_t) in voluntary contraction at different loadings of the muscle.

The figure shows that for each of the forces the frequency of the electromyogram rises linearly along with the increase in the muscle shortening. The figure depicts a result that is in agreement with that obtained in the study of JALAVISTO *et al.*, referred to above (crosses, broken line), which is clearly analogous with that obtained by us. In the table attached to their study the values of one experimental series only (loading about $3 \cdot 10^5$ dyne) were indicated. They are nevertheless comparable with our results as the experiments were carried out with the same muscle and using the same method. The slight discrepancy between our results (squares) and those obtained by JALAVISTO *et al.* (crosses) may be attributable to differences in the method by which the impulse frequency was estimated (see the work referred to above and BERGSTRÖM 1959 a). The figure also indicates that an increase in the muscle force is accompanied by an increase in the impulse frequency.

It is also evident from Fig. 3 that the curves depicting the results intersect the vertical axis at frequencies, which are descriptive of the electric activity of the muscle at its rest length $\triangle s = 0$, the forces produced by the muscle being $k = 1.69 \cdot 10^5$, $3.38 \cdot 10^5$ and $6.76 \cdot 10^5$ dyne, respectively. Since it may be assumed that the proportionality between the impulse frequency of the muscle and the shortening of the muscle also retains its validity for muscle lengths greater than the rest length (the value $\triangle s = 0$ in Fig. 3), the results presented in Fig. 3 make it possible to determine, in accordance with Fig. 4, the theoretical maximum length of the muscle, whose corresponding impulse frequency is $\nu_t = 0$. The Fig. 4 has been obtained so that the empirical curves of Fig. 3 (b, c, d) have been continued so as to intersect the horizontal axis. The mean value for these intersepts has then been taken as the "theoretical" 0-value, $\triangle l = 0$. Proceeding like this, the value of the muscle shortening

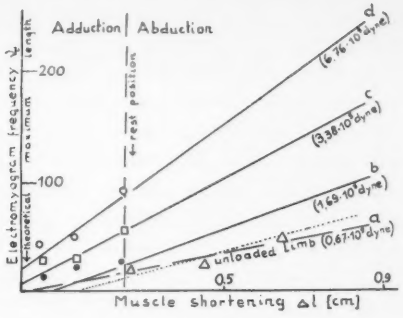

Fig. 4. Shortening of m. interosseus dors. I estimated from the "maximal length" corresponding to motor frequency $\nu_I = 0$ ($\triangle 1$), and the frequency of the motor discharge (ν_l) in voluntary contraction at different loadings of the muscle and with an unloaded muscle.

corresponding to the rest position of the finger was found to be $\triangle l = 0.25$ cm (the "rest position", in Fig. 4).

In order to find empirical support to the theoretical parts of the curves (b, c, d), to the left to the vertical axis, experiments similar to those performed by the abducted finger were also carried out with an adducted finger. The results (means of ten recordings) are represented by the hollow circles and squares, and the black circles to the left of the vertical axis in Fig. 4. The standard deviations were of the same order of magnitude as in the results shown in Fig. 3. The degree of adduction corresponding to the value $\triangle l = 0$ could be reached with no subject, owing to the limits set by the proximal joint of the finger. The results seem to support the theoretical curves, even though the frequencies near the point of intersection (the value $\triangle l = 0$) were larger than assumed. As can be judged from the Fig. 1, the large ν_l-values may be due to the fact that in maximal adduction the muscle is pressed against the os metacarpus, with the result that the conditions of contraction are changed.

In order for us to be able to determine the interdependence of the impulse frequency of the muscle and the muscle shortening also with unloaded finger (*i. e.* in cases where the load only consists of the finger's own mass), experiments were carried out so that the subject was permitted to perform liminal abductions, at different degrees of abduction, in which the finger made a minimal abduction movement just discernible with the eye. The action currents of the abductor muscle were recorded simultaneously and the impulse frequency was determined. It turned out that in most cases there were 2 separate impulses in the electromyogram, more rarely 1 or more than 2. The frequencies obtained are represented by the white triangles and the broken line (curve a) in Fig. 4 (means of ten recordings; the standard deviations of the same order of magnitude as above). It is justifiable to assume that the result obtained is descriptive of the inter-relation of the impulse frequency and the muscle shortening in cases where the finger is unloaded, for an extrapolation of the

curves b, c, d to the load 0 dyne yields almost the same result (the dotted line in Fig. 4). Under the experimental conditions represented by the broken line, the force at the insertion point of the abductor muscle, produced by the muscle, can be determined by determining the force acting upon the forefinger (at a distance of 7 cm from the abduction axis and in the direction of the abduction) which is sufficient to bring about a just discernible abduction. This "inertial" force of the finger was 10 g on the average, which is in agreement with the result obtained in the above study of JALAVISTO *et al.* At the distal insertion point of the muscle this force corresponds to $k = 0.67 \cdot 10^5$ dyne.

Although no work was done by the finger in our experiments, since the finger was at rest in a desired degree of abduction when the finger was loaded and the recordings were carried out, it is possible nevertheless to estimate from Fig. 4 that muscle work (A) which it has been necessary for the muscle to do in order to attain the abduction degree in question. Fig. 5 represents the interdependence of the muscle work, determined from the results shown in Fig. 4, ($A = k \cdot \triangle l$, where the muscle shortening $\triangle l$ has been estimated the "theoretical" maximum length of the muscle as the point of departure), and the impulse frequency of the muscle. Curves a, b, c and d, drawn through the zero point of the A-axis, are slightly divergent.

When the slopes, $H_a = A/v_f$, of the curves are computed from the results shown in Fig. 5 the average value found for curve a is $H_a = 1{,}200$, for curve b $H_a = 1{,}500$, for curve c $H_a = 1{,}820$ and for curve d $H_a = 2{,}150$ cgs units.

Discussion

It was observed that, under the experimental conditions concerned, the work done by the abductor muscle (m. interosseus dors. I) in contraction and the degree of shortening of the muscle were both directly proportional to the impulse frequency of the action currents, recorded from non-selective surface electrodes and measured at the terminal of the muscle shortening. The experimental result is in agreement with that of JALAVISTO *et al.* (1938), according to which an isotonic shortening of the muscle is accompanied by an increase in the said frequency.

The experimental results may be formally represented as

(1) $$A = k \cdot \triangle l = H_a \cdot v_f, \ (H_a = \text{const.}),$$

where A stands for the mechanical work done by the muscle in contraction, k for the muscle force, $\triangle l$ for the muscle shortening and v_f for the total frequency of muscle currents, measured at the terminal point of the muscle shortening. H_a, dependent of the load, is a coefficient of proportionality, which has the dimensions of physical action (energy · time, Germ. Wirkung), *i. e.* $g\ cm^2\ s^{-1}$. Equation (1) is comparable with the result of one of our previous studies (BERGSTRÖM 1959 b), according to which the energy (E) brought by the muscle in voluntary contraction to its environment (this study also dealt

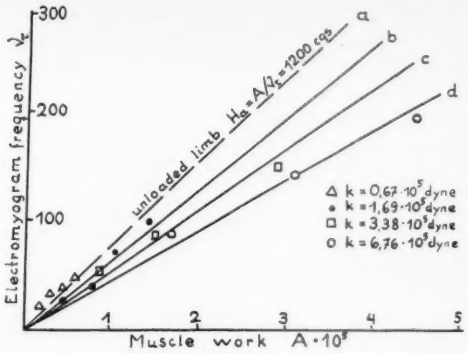

Fig. 5. Interdependence of the muscle work done by m. interosseus dors. I in voluntary contraction (A) and the frequency of the motor discharge (ν_t). k = loadings of the muscle.

with the abductor of the forefinger) was directly proportional to the total frequency of the muscle action currents (ν_t),

(2) $$E = H_e \cdot \nu_t, \quad (H_e = \text{const.}).$$

In this equation the coefficient H_e was shown by the experiments to be a constant and had the dimensions of action ($g\ cm^2\ s^{-1}$).

As is evident from equations (1) and (2), the impulse frequency appears as an energy parameter in both cases. In equation (1) it is descriptive of mechanical work, which is independent of time, and in equation (2) of the energy deriving from the state of motion of the muscle, which is accordingly dependent on time. Since the frequency magnitude involved in the equations is descriptive of the same state of a motor nerve (and muscle) in both cases, the mechanical work and kinetic energy must be here, as far as we can see, closely related with each other. To be able to comprehend this relationship and the relationship obtaining between the empirical events represented by equations (1) and (2), we propose to make the experimental results shown in Fig. 4 the subject of a more detailed scrutiny.

As the voluntary control of the adductor of the forefinger may be assumed to be similar, in point of principle, to that of its abductor, the results set out in Fig. 4 also allow of a representation analogous with that used in Fig. 6. In this representation the horizontal coordinate (l) represents the change in the position of the finger (described in terms of the muscle shortening) with respect to the space of its environment, which is brought about by the adductor-abductor system, and the vertical coordinate the period frequency (ν_T) of the abductor corresponding to each particular position. Since, according to our experiments, to each l-shift in the coordinate system (ν_t, l) there corresponds a given ν_t-shift, then, by virtue of equation (2), there also corresponds to it a given change in the energy of the system, $E = H_e \cdot \nu_t$. This change of energy

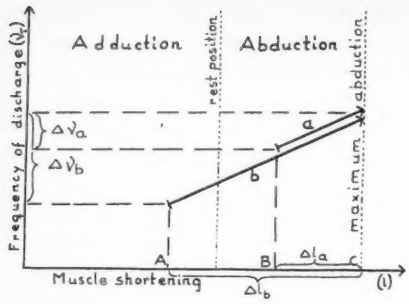

Fig. 6. Movement of the adductor-abductor system of the forefinger and the frequency of the motor discharge. (l) = shortening of the abductor muscle. Movements of the system indicated by the vectors $\vec{a}$ and $\vec{b}$.

may obviously be brought about by any change in the position of the system whatsoever, whether it be effected by the abductor or the adductor. In Fig. 6, two contractions of the abductor (vectors $\vec{a}$ and $\vec{b}$) have been represented, the length of the muscle after the contraction being the same (maximal abduction, say) in both cases. The l-projections of the contraction indicate the muscle shortenings and the ν_t-projections the changes in the impulse frequency of the abductor, to which there corresponds, according to equation (2), a well-defined change in the energy $E = H_e \cdot \nu_t$. The energy of the contraction $\vec{a}$ may be increased so that the adductor is made to stretch the abductor (from the l-value B to the l-value A), in which case a corresponding amount of energy, $E = H_e \cdot \nu_t$, may be freed in contraction $\vec{b}$. The amount of energy, $H_e \cdot \nu_t$, involved in the stretching, may be regarded as the "potential energy" which is "stored" into the stretch. This storing is carried out by the adductor, in which there has been a corresponding shortening and an increase in ν_t, and which has consequently lost energy.

This is to say that the energy (work) freed by the muscle in contraction is the larger, the larger is the initial length when the muscle is shortened into a specified terminal length.

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The Electrical and Mechanical Activity of a Muscle in Voluntary Contraction

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Abstract

BERGSTRÖM, R. M. The electrical and mechanical activity of a muscle in voluntary contraction. *Acta physiol. scand.* 1959. *47.* 199—209. — The voluntary control of the energy consumption of the muscle is, according to our previous studies, reducible to the number of time periods of the action currents fired into the muscle. This number of periods defines the physical action (energy · time, $g \cdot cm^2 \cdot s^{-1}$) carried by the muscle into the environment during contraction. As the physical action corresponding to one time period can be regarded as constant, the electrical and mechanical phenomena in the muscle can be represented satisfactorily in terms of a vector coordinate system, whose dimensions are the number of discharged motor time periods and the temporal and spatial shifts of the muscle movement. The interrelations of these dimensions were examined in voluntary actions of the abductor of the forefinger (m. interosseus dors. I) in human. The muscle action currents were recorded from non-selective electrodes. It was observed that the duration of contraction and the muscle shortening were both, within certain limits, directly proportional to the number of periods of the motor discharge.

It was previously found that the kinetic energy that a muscle carries into the environment in connection with voluntary contraction is proportional to the impulse frequency of the action currents of the whole muscle (BERGSTRÖM 1959 b). This relation was expressed formally as

(1) $$E = H_e \cdot \nu_i \quad (H_e = \text{constant}),$$

where E stands for the energy produced by the muscle, ν_t ($= dn_t/dt$, n_t being the number of time periods in time t) for the total impulse frequency of the muscle, and H_e is a constant which, if the proportionality is expressed in the form of an equivalence, has the dimensions of physical action (energy · time), *i. e.* $g \cdot cm^2 \cdot s^{-1}$. This relation was also written in the form

$$H_t = \int_{t_1}^{t_2} E \cdot dt = n_t \cdot H_e, \tag{2}$$

where H_t stands for the physical action performed by the muscle and n_t for the number of time periods (τ) of the motor discharge in time t. H_e is the same constant as in equation (1). It is provided that $n_t \cdot \tau = t$ and $\nu_t = 1/\tau$.

We have also investigated (BERGSTRÖM 1959 c) the relationship between the work (A) done by the muscle in voluntary contraction and the impulse frequency (ν_t, measured at the terminal point of the muscle shortening) of the muscle action currents. The result was presented formally as

$$A = k \cdot \triangle s = H_a \cdot \nu_t \quad (H_a = \text{constant}). \tag{3}$$

Here k is the muscle force, $\triangle s$ the muscle shortening and H_a a constant, which is of the same dimensions and of the same order of magnitude as the constant H_e in equations (1) and (2). This relation indicates that as an energy parameter the motor impulse frequency seems to be independent of whether the contracting muscle possesses kinetic energy, which is dependent of its state of motion or whether it does work, which is independent of time.

The voluntary gradation of muscle contraction is reducible to the number of time periods (n_t) of the motor discharge, which is dependent on the number of recruited motoneurones, their impulse frequency (ν_t) and the duration (t) of this frequency ($n_t = \nu_t \cdot t$) (ATKINSON, BROWN and GESELL 1940, GELLHORN 1953, BERGSTRÖM 1959 b). On the other hand is, by equation (2), the mechanical effect of the muscle dependent on this number of time periods. If the muscle energy is based on the constancy of energy corresponding to one time period, *i. e.* on the constancy of H_e in accordance with equations (1) and (2), the electrical end mechanical process of voluntary contraction in the muscle can be expressed in terms of the temporal and spatial dimensions and the number n_t.

The objective of experiments to be described subsequently, in which the spatio-temporal relations of voluntary muscle contraction were compared with the number of periods of the muscle action currents, was to find such a representation of electrical and mechanical activity involved in voluntary muscle movement.

Methods

The present experiments, as the experiments of our studies referred to above, were carried out with the abductor muscle of the forefinger (m. interosseus dors. I). As regards the method and recording of the muscle action currents, as well as that of

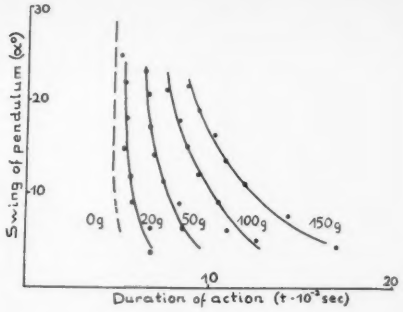

Fig. 1. Relationship of the duration (t) of the voluntary abduction of the forefinger and the amplitude ($a°$) of the swing of the pendulum, caused by the abduction, at different pendulum loads. The broken curve is descriptive of the result found by extrapolation for the load = 0 gr.

estimating the number of time periods from the gross response electromyogram, we refer to the previous works (BERGSTRÖM 1959 a, b, c). The determination of the duration of the action was carried out electrically, direct on the recording film. The muscle shortening was estimated from the abduction angle of the forefinger by taking preliminary X-ray photographs of the hand of the subject at different degrees of abduction of the forefinger (BERGSTRÖM 1959 c).

To determine the kinetic energy the abduction movement of the forefinger was transmitted to a pendulum, the physical properties of which were known (BERGSTRÖM 1957). Abduction took place in the horizontal plane, the flat of the hand being supported by a plaster mould. The stretched forefinger was fixed to a light, moving rail, whose axis of motion coincided with the axis of abduction.

In an other type of experiments the loading of the abduction movement of the forefinger took place with the aid of hanging weights (0—150 g), the force of which was transmitted to the distal joint region of the finger (BERGSTRÖM 1959 c). The 8 subjects were mostly the same as in the studies referred to (BERGSTRÖM 1959 b, c), so that they were familiar with the task in question. An attempt was made to avoid muscle fatigue in all experiments.

Results

Fig. 1 indicates how the time t, taken to perform a voluntary abduction of the forefinger, depends on the strength of action. The strength is represented by the amplitude a of the swing of the pendulum arising from the stroke given by the finger. The various curves (each point represents the mean for 30 observations, the standard deviations varying between ± 0.002 and ± 0.011 sec) are descriptive of the results obtained with different weights of the pendulum: no additional weight = effective weight of 20 g and additional weights of 50, 100 and 150 g. From the figure it is evident that an increase in the strength of action is accompanied by a decrease in the time taken to give the stroke and that an action with an unloaded limb (broken line, find by extrapolation from the empirical curves) happens in conditions where t is constant. As the actions were performed in natural circumstances, *i. e.* the only task the subject had to perform was to give strokes of varying strength to the pendulum, this time-constant way of acting of the unloaded

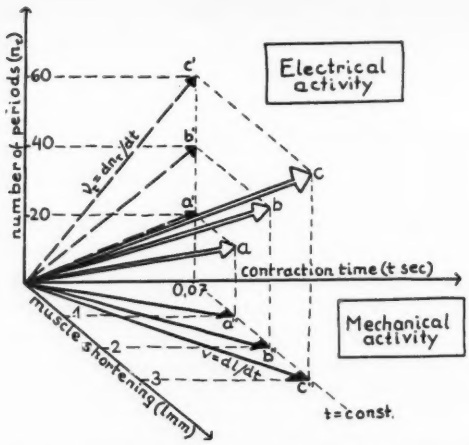

Fig. 2. The relationship between the time of contraction (t) of the abductor of the forefinger, the muscle shortening (l), and the number of periods (n_T) of the electromyogram in the isotonic unloaded voluntary abduction of the forefinger. The white arrows a, b and c are descriptive of actions, whilst their projections in the (n_t, t)-plane represent the electrical process, and their projections in the (l, t)-plane the mechanical process in the muscle.

limb can be considered a normal physiological phenomenon in the voluntary regulation of the action strength. When the pendulum load is increased, an ever more accentuated shortening of the action time accompanies the increase in the strength of the stroke.

Fig. 2 is a graphical representation of the temporal and the muscle-shortening relationships of the isotonic abduction of the forefinger, considered as a function of the number of periods of the recorded muscle action currents. The result represents a typical series. The representation is three-dimensional, the dimensions being n_t = the number of time periods in the electromyogram, t = the time taken to perform the action and l = the muscle shortening during the action. The events represented in the (n_t, t)-plane are descriptive of the electrical activity in the muscle and, in consequence, in the motor nerve in voluntary action. The events in the (l, t)-plane represent the mechanical motion of the muscle with respect to the environment.

In the voluntary contractions depicted in Fig. 2 the action time t remains constant as the unloaded muscle shortening increases. Inspection of the figure reveals that the number of periods (n_t) in the electromyogram increases in direct proportion to the muscle shortening (l).

The vectors $\vec{a}$, $\vec{b}$ and $\vec{c}$ (white arrows) are descriptive of the voluntary contractions. Their projections $\vec{a}'$, $\vec{b}'$ and $\vec{c}'$ on the (n_t, t)-plane (black arrows, broken lines) indicate electrical phenomena in the muscle and nerve: the

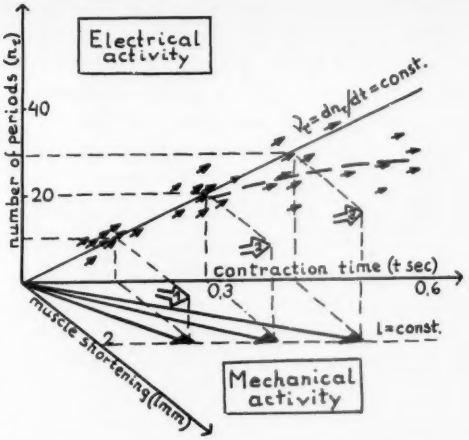

Fig. 3. The influence of the duration (t) of the voluntary abduction of an unloaded forefinger upon the interdependence of the abductor shortening (l) and the number of periods (n_t) of the muscle action currents according to a typical series of experiments. The black arrows in the (n_t, t)-plane represent individual recordings in abductions where the muscle shortening was constant.

tangent dn_t/dt of the projection curve describes the period frequency $\nu_t = dn_t/dt$ of the action currents. This tangent, determined at the terminal point of the muscle shortening (the black arrowheads on the (n_t, t)-plane), increased in direct proportion to the muscle shortening. The projections $\vec{a}''$, $\vec{b}''$ and $\vec{c}''$ of the vectors $\vec{a}$, $\vec{b}$ and $\vec{c}$ in the (l, t)-plane (black arrows and continuous lines) are descriptive of the mechanical process in the muscle with respect to its environment. In this case the tangent $v = dl/dt$ of the position vector indicates the rate at which the muscle shortens.

Fig. 3 shows the effect of the voluntarily prolonged contraction time t upon the interrelations of the number of periods n_t and the muscle shortening l in the same case of an unloaded finger. The black arrows on the (n_t, t)-plane of the coordinate system represent the results of a typical series of actions of the type described by the white arrows 1, 2 and 3. The muscle shortening l in these actions remains constant. It is seen from the figure that for small values of t the tangent of the (n_t, t)-projection of the actions 1, 2 and 3 (continuous line on (n_t, t)-plane as mean result) is constant. This is to say that for small values of contraction time (t) the period frequency (ν_t) of the muscle action currents is constant ($\nu_t = dn_t/dt = \text{const.}$) when the mechanical work done by the contraction remains constant ($A = k \cdot l = \text{constant}$; A = muscle work, k = muscle force, l = muscle shortening).

It can be observed, however, from the Fig. 3, that the tangent (ν_t) tends

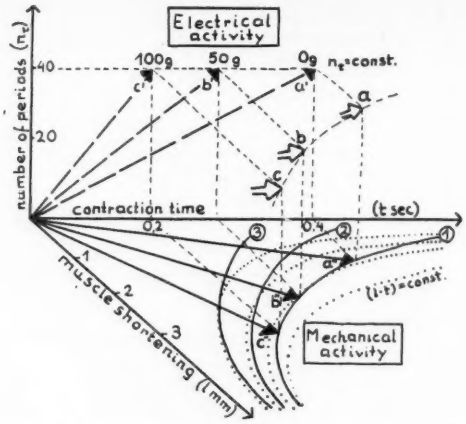

Fig. 4. The relationship of the duration (t) of voluntary contraction of the abductor of the forefinger, loaded with masses of various magnitude, and the muscle shortening (l) in actions with a constant number of periods (n_t) of the motor discharge. The white arrows a, b and c represent typical actions for loads 0, 50 and 100 g. The dotted curves in the (l, t)-plane are theoretical curves for the case $l \cdot t$ = constant. The continuous curves (1, 2 and 3) represent observational results.

to decrease when the contraction time is prolonged (broken line on the (n_t, t)-plane). It should be noted that the tangent $v = dl/dt$ of the (l, t)-projections of the contractions 1, 2 and 3 also decreases, when the contraction time is prolonged. This indicates a decrease in the speed of the contraction and correspondingly a decrease of the kinetic energy produced by the contraction.

Results of experiments in which the abductor of the forefinger was loaded in voluntary actions, with weights of differing magnitude, are seen in Fig. 4. Three actions (the white arrows a, b and c) are depicted. The number of periods of the muscle action currents is the same for each of these actions, the load varying from 0 to 100 g (at the distal phalanx of the finger). Inspection of the (n_t, t)-projections reveals the increasing effect of the additional loads upon the discharge frequency $\nu_T = dn_t/dt$.

In the (l, t)-plane of the Fig. 4 we have drawn theoretical curves (dotted lines) in which $l \cdot t$ = constant, *i. e.* for which the product of duration of contraction (t) and the muscle shortening (l) is constant. As is evident from the figure, the actions a, b and c for which the number of time periods (n_t) is the same, are almost ($l \cdot t$)-constant. The continuous lines 1, 2 and 3 in the (l, t)-plane are the loci of the (l, t)-projections of the termini of muscle contractions of various strengths. These lines are expressed as average values obtained from experiments. Each of them is obtained as the mean of 22 to 40

recordings, the standard deviations varying between $\pm$ 0.007 and $\pm$ 0.018 sec. The figure shows that the empirical curves 1, 2 and 3 (for each of which n_t is constant, *i. e.* for curve 1: $n_t = 43$, for curve 2: $n_t = 31$ and for curve 3: $n_t = 18$, with a maximal variation of 4 periods) follow the course of the theoretical (dotted) curves the closer, the shorter is the duration (t) of the action. When the duration is increased and especially when the muscle contraction approaches the isometric type, the empirical curves diverge from the theoretical ones.

Discussion

The experiments reported above were concerned with voluntary muscle action (the abductor of the forefinger). The relation of the duration of contraction and the muscle shortening to the number of time periods of the action currents, recorded from the whole muscle, was examined. In preliminary experiments, where the durations and the muscle shortenings in muscle actions of varying strength were measured, it was found that when the subject increased the strength of the action of an unloaded limb, this took place by increasing the muscle shortening, whilst the duration of the action remained the same. This may be interpreted to mean that the natural way for the human to gradate the strength of the action, in the case of an unloaded limb, is to gradate the physical work done by the muscle. The magnitude of the work depends, in the isotonic contraction concerned, upon the muscle shortening only. As the action time remains constant, we are concerned with work which is "independent" of the time taken to perform it.

In comparing the number (n_t) of time periods of the electromyogram to the muscle shortening (l) in this kind of actions of an unloaded limb, they were found to bear a linear relationship to each other. This is in agreement with the results of one of our earlier studies (Bergström 1959 c), according to which the work $A = k \cdot l$ (k = muscle force) done by a voluntarily acting muscle and the period frequency (ν_t, measured from the terminus of the shortening), attained in the muscle during work, bear a linear relation to each other in accordance with equation (3) $A = k \cdot l = H_a \cdot \nu_t$, where H_a = constant. In the case now under consideration (unloaded limb) the number of periods, n_t, depends on the period frequency alone, since the time t during which the frequency is effective, is, as was presented above, a constant. This is comprehensible from the interrelation obtaining between these magnitudes, $n_t = \nu_t \cdot t$.

This result accordingly lends support to the conception, that in voluntary action the central nervous system gradates the mechanical effect of the muscle through the number (n_t) of time periods ($\tau = t/n_t$) it fires to the muscle. This number of time periods is, in physiological regard, dependent on the recruitment of the motoneurones, their action frequency and the duration of their activity ($n_t = \nu_t \cdot t$).

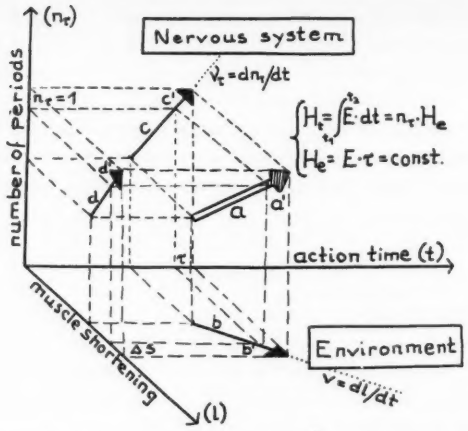

Fig. 5. The representation of voluntary muscle action ($\vec{a}$) in the form of a geometrical vector manifold in a coordinate system whose dimensions are the number of periods (n_t) of the motor discharge, the duration of muscle contraction (t) and the muscle shortening (l). Vector $\vec{a}$ represents a muscle action made up of unit vectors $\vec{a}'$ corresponding to a time period τ. The projection $\vec{c}$ is descriptive of the periodical processes of the motor nervous system and projection $\vec{b}$ of the mechanical processes of the muscle in the environment.

If account is taken of evolutional points of view, we may start in muscle physiology with the assumption, that the regulation of voluntary muscle action is originally (in childhood) adapted to the directing of the movements of an unloaded limb, *i. e.* the motion is isotonic, the load consisting of the constant mass of the limb alone. As can be judged from a certain developmental stage of the child, the loading of movements with the "foreign" masses of the environment is a phenomenon which makes its appearance only later on. In this sense, voluntary directing of movements within the framework of these masses must also be "learnt". From this point of view, our experiments with an unloaded finger represent the basic type of the voluntary control of muscle movements. This basic type of central nervous control accordingly consists in the gradation of the muscle shortening (l), *i. e.* muscle work ($A = k \cdot l$), the force (k) being constant. This gradation takes place within a constant time, with the aid of the number of time periods of the motor discharge, (n_t), directly proportional to the muscle shortening,

$$A = k \cdot l = n_t \cdot H_a \text{ (for } t = \text{constant}). \tag{4}$$

The time taken to perform the muscle work, provided that it is not long, does not cause any exceptions to this rule (figure 3), the same being true of the load (Fig. 4) for movements of short duration.

From equation (3), $A = k \cdot l = H_a \cdot \nu_t$, we may derive formally (taking into account that $\nu_t = dn_t/dt$)

$$A \cdot t = k \cdot l \cdot t = n_t \cdot H_a. \tag{5}$$

On the basis of this equation, which involves magnitudes presented above, it is possible to assume that the product of the muscle force (k), the distance of its action (l) and its action time (t) is proportional to the number of time periods (τ) of the motor discharge. According to the experimental results presented in Fig. 4 this is actually the case, and the more accurately so, the smaller is the variability of the duration of the voluntary movement of the muscle.

What has been presented above serves to indicate that the temporal and spatial relations of muscle actions are, within certain limits, functions of the number of time periods of the motor discharge.

This is to say that the phenomenon of voluntary muscle contraction allows a representation in terms of a three-dimensional manifold, in which the variables are the time (t), the location (l) and the number of periods (n_t). In the Fig. 5 the vector $\vec{a}$ (white arrow) showing the muscle contraction is represented in the (n_t, l, t)-coordinate system. The (n_t, t)-projection $\vec{c}$ of this vector represents the gradating electrical processes of the motor nervous system. The slope of the projection $\vec{c}$, $dn_t/dt = \nu_t$, expresses the impulse frequency of the motor discharge. The (l, t)-projection $\vec{b}$ of vector $\vec{a}$ represents the mechanical event of the muscle in the environment. The slope $ds/dt = v$ of this projection indicates the velocity of the limb-muscle system.

According to the equations (1)—(5) the mechanical events in the muscle are in the (n_t, l, t)-system related to the electrical processes in the muscle and the motor system. So is by equation (2), $H_i = \int_{t_1}^{t_2} E \cdot dt = n_t \cdot H_e$, the physical "action" (energy · time, $g \cdot cm^2 \cdot s^{-1}$) of the voluntary muscle contraction determined by the n_t-projection of the vector $\vec{a}$.

Inspection of the (n_t, l)-projection $\vec{d}$ of vector $\vec{a}$ reveals that the muscle contraction allows a geometrical representation in the l-dimension. This l-dimension is structured into parts ($\triangle s$), defined by the time periods (τ) of the motor discharge. The physical action (energy · time) corresponding to such a part is, by equation (2)

$$H_i = E \cdot \tau = H_e = \text{constant}, \tag{6}$$

since $n_t = 1$ (BERGSTRÖM 1959 b). This "unit action" H_e (energy · time period), involved in the path of voluntary muscle movement, can be (within certain limits) empirically determined from the relationship obtaining between the electrical and mechanical activity of the muscle (BERGSTRÖM 1959 b, c). In

Fig. 5 the distribution of this "unit action" in the l-dimension is represented by the vector $\vec{s}$, which is the l-projection of $\vec{a}'$, corresponding to $n_t = 1$.

When an unloaded limb is active obeying the principle t = constant, the geometrical mode of representation on the (n_t, l)-plane of the (n_t, l, t)-system is very suitable for this "basic type" of voluntary gradation of muscle movements. This manner of muscle action suggests that the geometrical representation is natural for muscle activity. Thus the neuro-muscular phenomenon involves this "geometry" as an integral-numbered structure typical of itself, in which the physical action (H_t) is expressible as a dimensionless number n_t.

The question as to whether the above geometrical mode of representation of neuro- and muscle-physiological processes is advisable from the physiological point of view is to be decided, in our opinion, by practical considerations. It seems to us, however, that it is capable of giving a more real meaning to the parameters descriptive of neurophysiological processes in that it associates them with the physical world of our external environment in accordance with equations (1) and (2). Moreover, it is necessary for the development of neurophysiology from a branch of physiology dealing with a pure "signal system" into a branch of physiology concerned with the energetic interaction between the internal and the external environment of the organism, that the parameters descriptive of neural processes be connected with those descriptive of the physical phenomena of the environment.

It should be noted, however, that our equations represent only formal expressions of processes involved in the voluntary muscle contraction. Thus the causal relationship between the electrical and mechanical parameters in these equations forms a special problem, in which the physiological process described by the factors H_e and H_a is of a primary importance.

From the point of view of general sense physiology, the problem setting of which (see REENPÄÄ 1959) has led us to the reported experiments and structure description of voluntary muscle activity, our mode of presentation is a necessity. From this point of view, the causal relationship of the processes represented by equations (1) and (2) do not form a criterion of the theory, either, since what is concerned is the representation of structures in terms of different conceptual manifolds (in our case within the realm of the electric processes of the nerve and muscle and of the mechanical processes of the muscle). The criterion of this description is, according to the sense physiological principles, the "subjective" perception concerned with these quantities, the structural properties of which are obeyed by our conceptuality (REENPÄÄ 1953, 1959, BERGSTRÖM 1957).

Within the framework of this criterion, the physiological process of voluntary muscle action, discussed in this paper, does not form any exception. Even here, in voluntary actions, the perceptual component entailed in the total action concerned determines in the last analysis the mechanical result of the action. In voluntary activity this "perceptual" component is called "central

control". — These are the reasons why it is necessary to associate expressions descriptive of neurophysiological processes to those descriptive of processes in the environment.

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Total Hemoglobin in the Chick Embryo Developing in Different Oxygen Tensions

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Abstract

ASTOLA, E., E. JALAVISTO, O. MUSTALA and L. SOLANTERÄ. Total hemoglobin in the chick embryo developing in different oxygen tensions. *Acta physiol. scand.* 1959. *47.* 210—217. — The total hemoglobin of chicken embryos incubated 3—11 days in varying oxygen pressures (15—17, 21 and 40—50 per cent oxygen) and in different temperatures (38° and 40° C) was determined with the aid of a benzidine-peroxidase-reaction. In the youngest embryos the size of the area vasculosa was measured, and embryos more than 4 days old were weighed. It could be shown that the amount of hemoglobin was approximately one per cent of the weight of the embryo whatever the conditions. However, although the hemoglobin formation obviously did not respond to low oxygen pressure, the area vasculosa reacted to the lack of oxygen by increasing its size.

The mechanism of anoxic erythrocytosis probably gives the key for understanding of the fundamental stimulus of erythropoiesis. In spite of repeated attempts to elucidate this central question it has remained unsolved (GRANT and ROOT 1952). In the following the problem will be attacked from the point of view of developmental physiology. The reason for this approach lies in the fact that during embryonic development the different tissues and organs begin to differentiate and function in a particular sequence. Blood forming

fortunately begins very early and goes on throughout the whole embryonic and adult life. Adult birds react to decreased oxygen tension with erythrocytosis. If in the early chick embryo no signs of increased rate of erythropoiesis can be detected as a reaction to low atmospheric pressure the organism must gain this mode of reaction in some phase of its development. If the developmental stage at which the reaction appears is possible to ascertain, it might be possible to correlate this function with some of the organ functions or enzymatic differentiations appearing in the same phase of embryonic development.

Material and Methods

The experiments are from two periods, one in 1954—55 and the second one, the main part, is from 1958 and -59. In the orienting experiments the eggs (white Leghorn) were incubated in two nearly air-tight wooden boxes placed in a thermostat at 37° C. The boxes had in- and outlet tubes for flushing with different gas mixtures from pressure bottles. One of the boxes was filled with air, the other with a gas mixture of 40 % oxygen in nitrogen. The eggs were turned once a day whereby the boxes had to be opened. After closing the boxes, they were thoroughly flushed with air or the oxygen mixture, respectively. On the bottom of the boxes were placed two flat water containers for humidifying the air. After 3, 4, 5, etc. days the eggs were taken for analysis of hemoglobin.

Analysis of Hemoglobin. Bleeding of the embryo was carefully avoided and the vascular zone and the embryo were dissected out with as little albumen and yolk as possible. The embryo and the vascular zone were put into a test tube and diluted with an amount of distilled water necessary for homogenization. The final dilution varied according to the age of the embryo from 1 : 25 to 1 : 10,000. The determination of the hemoglobin content was performed according to a modification of the method of McFarlane and McKenzie Hamilton (1932). From the homogenate samples of 1 ml were placed in measuring flasks of 25 ml in which 2 ml of a 2 per cent solution of benzidine in 20 per cent acetic acid was placed. 1 ml of 0.6 per cent hydrogen peroxide was pipetted into the flasks and the developing colour reaction was allowed to proceed during 1 hour. The samples were then diluted to 25 ml with 20 per cent acetic acid and the colour intensity determined with a "Lumetron" filter-photometer at 550 mμ wave-length. The hemoglobin concentration was read from a standard calibration curve made of hen's blood, the hemoglobin content of the standard solution being determined in alkaline solution with an EEL colorimeter. When the dilution was only 1 : 25 and 1 : 50 there was some trouble with turbidity of the solutions, in the greater dilutions the final solutions were clear. Therefore in the main experiments 2—8 embryos were pooled whereby the dilution could be increased.

During the course of the experiments it became evident that the conditions of incubation were not as strictly controllable as would be desirable. Furthermore, it was noted that the hemoglobin standards had to be run parallel to the experimental analysis, the batches of benzidine reagent being too variable for use of a standard calibration curve. The experimental method was therefore modified as follows.

To ensure exactly the same temperature in the incubators with different oxygen tensions the eggs were placed in three glass jars of 3 l capacity immersed in the same water bath (Warburg apparatus). The jars were closed with a tight rubber plug, through which 2 tubes were inserted. On the bottom was a water layer above which lying on a perforated iron stand the eggs, 8—16, were placed. The gas mixtures were

introduced through a rubber tubing, the end of which reached the egg-stand. Thus the air came from below the eggs and thus secured an effective ventilation. The flow of the oxygen nitrogen mixtures was adjusted to approximately 300—500 ml/min and was going on as long as the incubation lasted. In addition to air which was pumped from room air mixtures of 15—18 and 40—50 per cent oxygen were used. The 15 per cent oxygen mixture was found to be on the limit of tolerance, since in embryos older than 7—8 days malformations of the nervous system were sometimes observed.

The temperature of the water bath was maintained at 38° C and 40° C (two Warburg's baths). During the course of the experiments it became evident that the time of incubation could not be used as a base of reference for varying amounts of hemoglobin formed in the embryos developing in different oxygen tensions. The speed of weight gain and (probably) the speed of embryonic differentiation were namely affected by the oxygen tensions. Therefore it was thought necessary to weigh the embryos. Since the weight of the embryo shows great interindividual variations the hemoglobin determination and the weighing had to be made on the same embryo. The embryo was freed from the embryonic membranes, put on a piece of filter paper and transferred quickly to the weighing cup which was stopped to avoid drying. The filter paper, the embryo and the membranes were put together for homogenization and determination of hemoglobin. Since a slight loss of hemoglobin is unavoidable, 2—8 embryos were pooled together whereby the loss becomes insignificant. The embryos younger than 4 days are so small that the weighing becomes very inaccurate. In the main experiments the youngest embryos, therefore, were 4 days old.

In the youngest embryos the size of the vascular area was compared with the amount of hemoglobin of embryos incubated in different oxygen tensions. An increase in the size of the hemopoietic area could possibly represent a mechanism of adaptation to hypoxia.

Results

Size of the area vasculosa in the early chick embryo. In the youngest embryos 64 and 67 hours old, the size of the vascular zone does not significantly differ in the eggs incubated in air and those incubated in 40 per cent O_2. The mean size of area vasculosa in the former is 112.8 ± 9.58 mm² and in the latter 92.1 ± 5.76 mm². Although a slight reduction of size in the 40 per cent oxygen eggs is discernible, it is approximately proportional to a slightly smaller content of hemoglobin in the embryos incubated in higher oxygen tension. The total hemoglobin values are namely in air: 16.6 ± 1.82 μg, in 40 per cent O_2: 11.5 ± 1.07 which makes 0.15 μg/mm², in eggs incubated in air and 0.13 μg/mm² in eggs incubated in 40 per cent O_2, respectively. In Fig. 1 a) the values for area and hemoglobin in the individual eggs are shown. When the age of the embryos increases the proportionality between size and hemoglobin content is, however, lost. As seen in Fig. 1 b) and c), beginning from areas about 300 mm², the hemoglobin values corresponding to those embryos, which have been incubated in 40 per cent oxygen are somewhat higher (broken lines) than those incubated in air (broken punctuated lines) or particularly higher than those incubated in 15—17 per cent oxygen (solid heavy lines). If only values belonging to the same batch of eggs and the same temperature

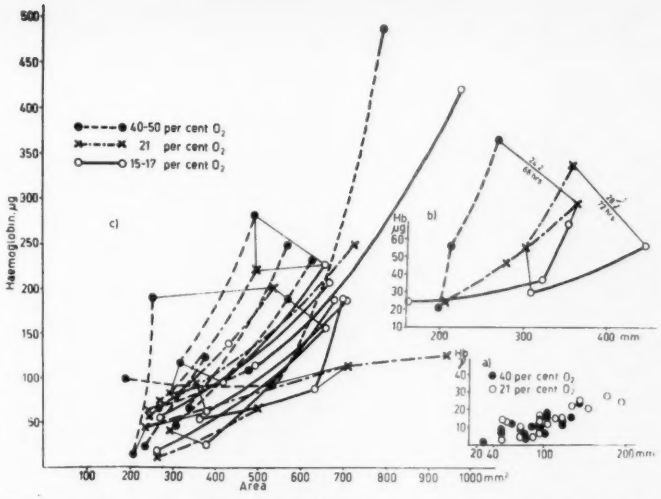

Fig. 1. Total hemoglobin of early chick embryos and area of the vascular zone.
a) Preliminary experiments. The determinations of hemoglobin, and of area are performed on the same, single embryo.
b) Pooled samples, main experiments. Large scale.
c) Smaller scale, all experimental values except those shown in b.

are compared (in the figure connected with light solid lines) the difference appears quite clearly: the 40 per cent embryos have more hemoglobin at the same area or even at a smaller area vasculosa than those incubated in 15 per cent oxygen. The values corresponding to incubation in air lie between the 40 per cent and 15 per cent values. Sometimes the area could not be measured. The average hemoglobin content occasionally does not correspond to the same number of embryos as the average size. This probably is responsible for part of the inconsistencies in the values. In the later, main part of experiments those embryos, in which the size of area vasculosa could not be measured, were discarded from the hemoglobin analysis; thus the size and hemoglobin always relate to the same embryos.

Total hemoglobin and weight of the embryo. In embryos more than 4 days old the size of the vascular zone is so great that it no longer can be measured. On the other hand only embryos 4 days old or older can be weighed with the necessary accuracy. If the weight is plotted against time of incubation, then variations in the humidity, aeration, temperature, and oxygen content greatly affect the results as shown by *e. g.* ROMANOFF *et al.* (1930—1938). Likewise the hemoglobin formation seems to be affected by these conditions. Since it is difficult exactly to control all these factors (except temperature) in different

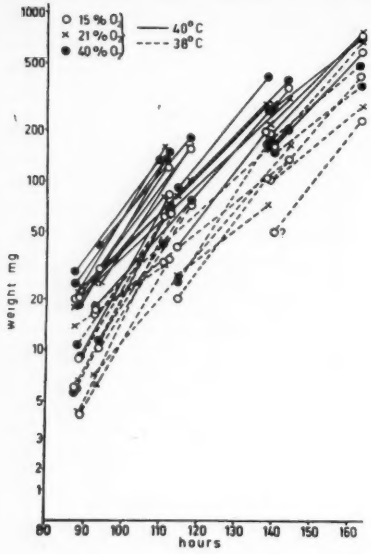

Fig. 2. Weight gain of chicken embryos under varying conditions of incubation.

incubation periods (Fig. 2) it seemed more appropriate to compare the hemoglobin content of embryos of the same weight. In Fig. 3 a) the total hemoglobin of young embryos (incubated during 94 hours) is shown. The total hemoglobin obviously increases linearly with the weight of the embryo the values for different oxygen tensions falling on the same line. A decreased oxygen tension obviously does not specifically stimulate the formation of hemoglobin. The hemoglobin content as percentage of body weight remains approximately one per cent notwithstanding differences in oxygen tension, temperature of incubation, 40° and 38° C. In Fig. 3 b) and c) hemoglobin content of older embryos is seen. The result is the same as with the younger embryos: the increase in total hemoglobin is roughly linear and proportional to the weight of the embryo without any difference according to oxygen tension or temperature of incubation. (In some experiments the values fall outside the main range but linearity with variations of weight and the independence of oxygen tension or temperature is conserved. The divergence is probably due to some accidental error in the hemoglobin standard rather than to a difference in the batch of eggs, although the last mentioned possibility cannot be ruled out either.)

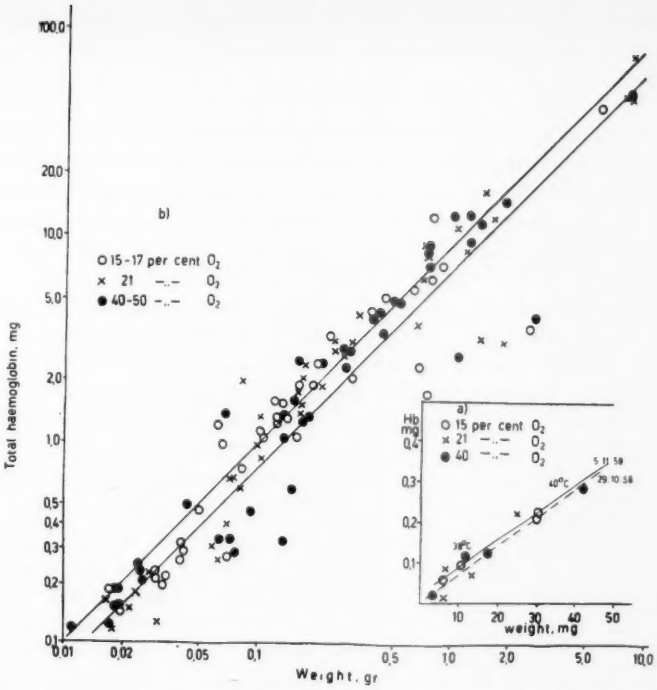

Fig. 3. Total hemoglobin as a function of embryonic weight. 4—11 day-old chicken embryos incubated under different oxygen tensions.

Discussion

The method used for determination of hemoglobin is a peroxidase reaction. Since it has been shown that egg albumen as well as many animal tissues contain peroxidases, it may be asked whether these sources may affect the values which we have considered as indicative of hemoglobin. Since admixture of albumen was avoided as much as possible and the dilution adjusted according to the amount of hemoglobin the intensity of reaction due to the hemoglobin must necessarily by far exceed that eventually arising from albumen. On the other hand in 8 day old exsanguinated embryos there are only traces of peroxidase demonstrable (BANCROFT and ELLIOT 1934); consequently tissue peroxidases cannot either affect the recorded values. The constant percentage of hemoglobin on weight basis may likewise be used as evidence for assumption that what has been measured is actually hemoglobin.

So far we know no direct measurements of the total hemoglobin in chick embryo exist. The hematocrit (YOSPHE-PURER *et al.* 1953, JOHNSTON 1955),

and the hemoglobin percentage of blood (ZORN and DALTON 1937, PILIPENKO 1957) has been determined but the last-mentioned not in embryos younger than 9 days old. The relative values on the 9—10th day were consistently between 5.2 and 6.9 per cent hemoglobin per 100 ml blood. If approximately 1 per cent of body weight is hemoglobin as indicated by our experiments and the hematocrit 20 per cent as found in several studies the volume of blood would be remarkably high but still quite reasonable and fairly constant, *viz.* 15.5 per cent of the body weight. The values 25 to 40 per cent obtained in determinations with micro-injections of Evans blue (YOSPHE-PURER *et al.* 1953, and SPRINGALL 1955) must be considered as most unlikely.

The average percentage of hemoglobin in packed red cells can be calculated when the total hemoglobin, the blood volume, and the hematocrit are known. The value, 32 per cent, would be fairly "normal" even for the red cells of mature animals. Thus the relatively great blood volume, 15.5 per cent, reflects the high water content of all embryonic tissues, whereas the red cell does not share this feature.

The effect of varying oxygen pressures on growth and hatchability of chicken embryos has been studied by CRUZ and ROMANOFF (1944) who state that there is an optimum approximately at 31—41 per cent oxygen; the rate is much slower at 21 per cent. At still lower oxygen tensions the weight gain is smaller and the hatchability much reduced, 11 per cent oxygen representing the limit of tolerance for 5 day old embryos. Our experiments were in accordance with their findings. No data on hemoglobin formation under the same conditions exist. FLEMISTER and CUNNINGHAM (1940) incubated eggs in normal and by 40 per cent increased atmospheric pressure and found a reduction in the hemoglobin percentage and the number of erythrocytes in the blood of embryos incubated at high pressure. (The youngest embryos studied were 8 days old.) They found, furthermore, that the allantoic vessels were less developed (stated by X-ray pictures after injection of thorotrast) in the embryos developing under increased pressure. In our experiments the smaller size of the area vasculosa in the young embryos as compared to their content of hemoglobin (and weight) is in accordance with the finding of a less developed vasculature in the allantoic sac.

It is rather interesting to note, that the synthetic mechanism responsible for the formation of hemoglobin does not respond to low oxygen pressure whereas the hemopoietically active area vasculosa does. Since in our experiments the reaction of the vascular zone to low oxygen tension appears during the somite stage and before hemopoiesis has begun in the spleen and bone marrow, it must depend on an only slightly, if at all, differentiated stimulus. The possibility that the decreased oxygen pressure would refrain the rate of hemoglobin synthesis but stimulate the rate of mitosis in the less differentiated mesenchymal cells, should perhaps be considered. If viz. hemopoiesis cannot go on at an equal rate in decreased oxygen tension, the only possibility of

compensation would be an increase in the size of the blood forming organ, *i. e.* in the size of area vasculosa. Whether the reaction of area vasculosa to low oxygen pressure has anything to do with the reactions seen in mammals after similar exposure is, however, difficult to know. Likewise it remains unsettled, whether there is some humoral agent released through the lack of oxygen which stimulates the growth of the vascular zone in analogy to the "erythropoietins" acting on blood formation in mature mammals after exposure to low pressure.

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Recycling of Intravenously Injected Palmitic Acid-1-C^{14} as Esterified Fatty Acid in the Plasma of Rats and Turnover Rate of Plasma Triglycerides

By

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Abstract

LAURELL, S. Recycling of intravenously injected palmitic acid-1-C^{14} as esterified fatty acid in the plasma of rats and turnover rate of plasma triglycerides. *Acta physiol. scand.* 1959. *47.* 218—232. — The disappearance of injected palmitic acid-1-C^{14} from plasma in the rat was followed. It was found that part of the complexity of the disappearance curves in experiments with starving rats resulted from exchange of non-esterified fatty acids (NEFA) between plasma and adipose tissue. The content of NEFA in adipose tissue was considerably higher in starving than in glucose fed rats. After a time lag part of the injected palmitic acid returned to plasma incorporated in triglycerides (TG) in starving as well as in glucose fed rats. The specific activity relationships between plasma TG and NEFA suggested that most of the NEFA in plasma was derived from plasma TG in the glucose fed rats and part of it in the starving rats. The ratio between the specific activity of plasma TG and of liver TG suggested that all plasma TG might be derived from liver TG in glucose fed rats. This was more uncertain in starving rats. The half life of endogenous plasma TG was found to vary between 3.0 and 8.5 minutes.

During the last few years a large number of papers have been published on the metabolism of plasma non-esterified fatty acids (NEFA). According to the concept that has crystallized out of these investigations, fatty acids leaving

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the fat depots are transported mainly as NEFA. Readers interested in a review of the subject are referred to FREDRICKSON and GORDON (1958 a).

In a previous publication (LAURELL 1959) studies were reported on the distribution of NEFA represented by palmitic acid-1-C^{14} after intravenous injection into rats. It was found that part of the palmitic acid injected returned to the plasma in esterified form, mainly as triglyceride (TG). A similar observation has also been made by FREDRICKSON and GORDON (1958 b) in Homo.

The present investigation is concerned with a more detailed study of the elimination of palmitic acid from the plasma and its recycling as TG in (a) glucose fed and (b) starving rats. It was found that the elimination curve for injected palmitic acid-1-C^{14} deviated from an exponential slope already before any significant activity began to appear in plasma TG. That the disappearance curve of NEFA radioactivity is complex was first observed by BIERMAN, SCHWARTZ and DOLE (1957) in experiments on dogs and rabbits. This subject has recently been discussed by FREDRICKSON and GORDON (1958 b) in connection with studies on human beings. Since observations made in the previous investigation (LAURELL 1959) had made it probable that the fat depots can take up palmitic acid without simultaneous esterification, it was thought worthwhile studying the relation between the specific activity of plasma NEFA and NEFA in the fat depots about 5 and 9 minutes after the injection of palmitic acid-1-C^{14}. It was hoped that this might permit an opinion whether the exchange of NEFA between plasma and fat depot pools was rapid enough to influence the elimination curve for palmitic acid-1-C^{14}.

The present investigation also included a study of the turnover rate of endogenous plasma TG. The only experiments hitherto made to determine this rate were recently published by BATES (1958) and were based on C^{14} decline after cross circulation between dogs that had received palmitic acid-1-C^{14} and unlabelled dogs. In that investigation, however, NEFA was included in the glyceride fraction determined and the curves from which she determined the turnover rate were extremely complex.

Experimental

The solution containing palmitic acid was prepared in the way described by LAURELL (1957). Palmitic acid-1-C^{14} was purchased from Amersham, England, and had a specific activity of 20 mc per mmole. The palmitic acid was dissolved in rat serum in a concentration of 0.16 to 1.15 μmoles per ml serum. White male rats weighing between 258 and 306 g were used. During the last 48 hours before the experiments the glucose fed rats were allowed to eat glucose and starch *ad libitum* but nothing else. The starving rats were deprived of food for 48 hours before the experiments. The animals were anesthetised with nembutal injected intraperitoneally as described earlier (LAURELL 1959). The palmitic acid solution was injected into a surgically exposed neck vein. The blood samples were collected from the other neck vein by means of a syringe and immediately injected into small ice-cooled centrifuge tubes containing EDTA-Na_2. As a rule, 0.4—0.6 ml blood could be withdrawn within 10—15 seconds. A larger blood

sample was drawn at the end of each experiment and used for quantitative determination of NEFA, TG fatty acids and phospholipid P.

Lipids in plasma, liver and fat depots were extracted in the way described previously (LAURELL 1959). The isolated fat samples were separated into phospholipid and non-phospholipid fats by means of chromatography on silicic acid columns according to BORGSTRÖM (1952 a). The non-phospholipid fractions were separated into NEFA and neutral fat according to BORGSTRÖM (1952 b) by means of extractions with alkaline ethanol but first with Amberlite IRA 400 in the case of lipids from fat depots. Lipids from livers and plasma samples intended for quantitative analysis were further separated into a cholesterol ester fraction and a TG fraction.

NEFA was dissolved in 1 ml ethanol after isolation and determined by titration. TG fatty acids were determined according to SCHMIDT-NIELSEN (1943). Phospholipid P was determined according to ALLEN (1940), and from this value phospholipid fatty acids were calculated.

Radioactivity measurements were carried out in a windowless gas-flow GM tube (background about 22 cpm). At least 1,000 counts were counted. All samples were plated on lens paper as described earlier (LAURELL 1959).

In order to determine the turnover rate of plasma NEFA the initial phase of the elimination of palmitic acid from the plasma was studied. 0.3 ml of rat serum containing 1 μc palmitic acid-1-C^{14} was injected into two starving and two glucose fed rats. Four samples were collected from each rat at 40 seconds intervals. Assay of radioactivity was made on extracted total fat corresponding to 0.05—0.2 ml plasma.

In experiments carried out to elucidate the exchange between NEFA pools in plasma and adipose tissue two glucose fed rats and two starving rats were used. 8.5 μc palmitic acid-1-C^{14} was dissolved in 0.5 ml rat serum and injected into a neck vein. Five and nine min later blood samples were drawn and most of the perirenal fat body on one side was removed, the latter procedure required 15 to 35 sec. The pieces of fat removed were homogenised immediately in chloroform-methanol. NEFA and neutral fat were isolated from the extract.

The later course of the elimination of the palmitic acid from plasma and its recycling as esterified fatty acid was followed for 2 hours after injection of 8.5 or 23 μc palmitic acid-1-C^{14} dissolved in 0.5 or 1 ml rat serum in two glucose fed and two starving rats. At the end of each experiment the liver was preserved for determination of specific activity in the glyceride and phospholipid fractions.

In the investigation of the turnover rate of endogenous plasma TG, 100 μc of palmitic acid dissolved in 1 ml 13 per cent human albumin was injected into a neck vein of glucose fed rats. The animals were bled about 40 min later and the blood allowed to clot at 4° C. 0.5 ml of the serum obtained after centrifugation was injected a few hours later into (a) glucose fed and (b) starving rats. The specific activities of NEFA and of the glyceride fractions were then followed in a series of plasma samples. A total of 3 series of experiments of this type were performed. The sera used for the injection contained 0.13—0.23 μc per 0.5 ml. Of this activity, about 1 per cent was found in the cholesterol esters, 57—75 per cent in TG, 13—30 per cent in NEFA and 10—12 per cent in phospholipids.

Results

Experimental determination of the turnover rate of plasma NEFA was difficult because the elimination curve for the palmitic acid deviated from an exponential slope already within 2 min. From the course of the curves during the first two minutes the half life was determined for non-esterified palmitic

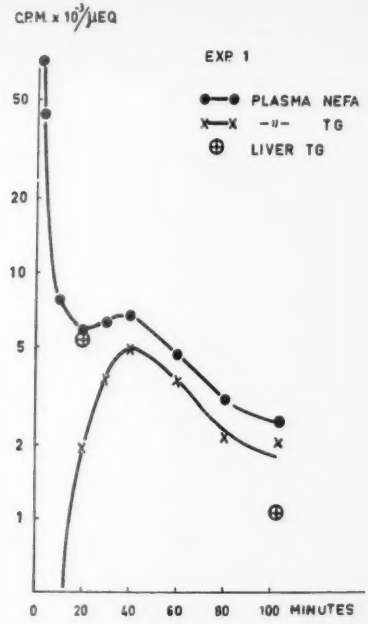

Fig. 1. Disappearance and recycling of injected palmitic acid-1-C^{14}. Glucose fed rats. Horizontal axis: min after the injection.

acid as 0.55 and 0.70 min for the 2 glucose fed rats and as 0.68 and 0.90 min for the starving rats. Corresponding means for turnover time for glucose fed rats was 0.90 min and for starving rats 1.15 min. These values are, of course, only approximate and may be regarded as an upper limit for the amount of NEFA that might be transported between different tissues.

Table I gives the results of experiments carried out to determine the exchange of NEFA between the NEFA pools in plasma and fat depots.

The results of the other parts of the present investigation are given in Fig. 1—6 and Table II. It is not quite apparent from Fig. 1 that the initial slope of the NEFA curve does not follow a simple exponential function in Exp. 1. The specific activities of NEFA obtained in this experiment were 401,000 cpm/μeq at 0.95 min, 72,000 at 2.1 min, 44,300 at 3.1 min and 7,700 at 10 min. The slopes between these points correspond to half lives of 0.5, 1.1 and 2.7 min, respectively. A deviation from an exponential course must thus have occurred already at 2.1 min.

Discussion

The purpose of glucose feeding and starving of the rats was to provide an experimental material representing two extremes with respect to fatty acid metabolism. In addition, this reduced the inflow of chylomicrons from the in-

Table I. Exchange of NEFA between the NEFA pools in plasma and adipose tissue (perirenal fat body)

Time min		NEFA cpm/μmole	NEFA per cent of total fat	NEFA meq/l
		Glucose fed rat no. I		
4.9	P	7,000		0.27
5.8	A	52	0.070	
9.1	P	5,000		0.27
9.8	A	37	0.066	
		Glucose fed rat nr II		
5.0	A	36	0.070	
5.9	P	7,800		0.20
8.8	A	38	0.067	
9.7	P	5,600		0.20
		Starving rat nr I		
5.0	A	2,020	0.23	
7.0	P	6,180		0.96
9.3	A	1,570	0.30	
10.3	P	3,910		0.96
		Starving rat nr II		
5.3	P	4,680		0.75
6.2	A	1,370	0.23	
9.2	P	1,680		0.75
10.8	A	950	0.22	

Time = minutes after injection of 8.5 μc palmitic acid-1-C^{14}
P = plasma sample
A = adipose tissue sample

testines to a minimum. It is therefore assumed that this fraction of exogeneous plasma TG is negligible and that the plasma TG under the experimental conditions, endogenous plasma TG, may be regarded as a single metabolic pool. This latter assumption is surely not strictly correct, but is in accord with the results obtained. The following discussion is simplified still further by neglecting the occurrence of mono- and diglycerides and by regarding the palmitic acid as representative of the entire NEFA-fraction, which, though not quite correct, is a reasonable approximation (FREDRICKSON and GORDON 1958 b). A steady state during the experiment is also assumed, the effect of the assumption on the reliability of the results being greatest regarding the NEFA pools.

Exchange of fatty acids between plasma and adipose tissue. It is clear from Table I that the specific activity of NEFA in perirenal fat of starving rats was high

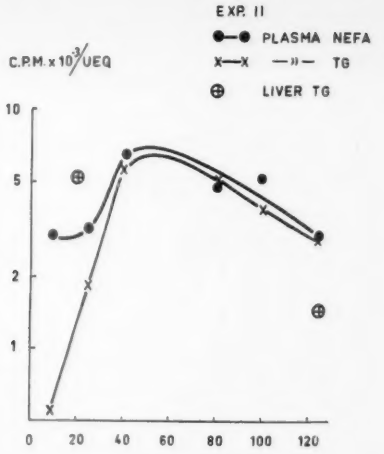

Fig. 2. Disappearance and recycling of injected palmitic acid-1-C^{14}. Glucose fed rats. Horizontal axis: min after the injection.

enough to influence the disappearance curve for palmitic acid-1-C^{14}. Provided that NEFA in the fatty depots may be regarded as a single pool, through which also fatty acids pass as they leave the fat depots, this pool would act partly as a dilution space for plasma NEFA. It should be observed that the specific activity and concentration of NEFA in the perirenal fat bodies is not representative of all fat depots. It was thus found in a previous investigation (LAURELL 1959) that 20 min after injection of palmitic acid-1-C^{14} the specific activity of neutral fat in this region was much lower than in other fat depots.

In contrast to what was seen in starving rats, the specific activity of the adipose tissue NEFA was very low in experiments on glucose fed rats. The activity noted in the adipose tissue NEFA in these experiments was even so low that it is possible that the major portion of it was due to the plasma content of the adipose tissue. Since about twice as much of the injected palmitic acid-1-C^{14} was incorporated in the fat depots of glucose fed rats as compared with starving rats (LAURELL 1959), these observations are somewhat surprising, and must imply a very rapid esterification of fatty acids taken up by adipose tissues under these circumstances.

As mentioned under Results, the elimination curve for palmitic acid deviated also in experiments on glucose fed rats from an exponential slope already before any significant activity had begun to appear in the plasma TG This makes it probable that a rapid exchange may occur between the plasma NEFA pool and pools other than adipose tissue NEFA without any corresponding net transport to or from plasma.

The amount of NEFA in adipose tissue may have been over-estimated by neglecting to consider the separation process in the determination of the blank for titration. Some readily hydrolysed lipid fraction might also have acted in

Table II. Turnover rate of plasma NEFA and TG

Exp. no.	NEFA			TG				TG turnover per cent of NEFA turnover
	μeq/ml	T min	Turnover rate μeq/min/ml	μeq/ml	T/2 min	T min	Turnover rate μeq/min/ml	
				Glucose fed rats				
5	0.49	0.68	0.72	1.12	3.5	5.0	0.22	31
6	0.32	0.68	0.47	0.99	3.7	5.3	0.19	40
9	0.33	0.68	0.49	1.23	8.5	12.2	0.10	20
10	0.24	0.68	0.35	1.36	7.5	10.8	0.13	37
								Mean 34
				Starving rats				
7	1.05	0.90	1.16	0.91	3.0	4.3	0.21	18
8	1.05	0.90	1.16	1.49	3.7	5.3	0.28	24
11	0.85	0.90	0.95	1.63	4.8	6.9	0.24	25
12	0.71	0.90	0.79	1.06	4.4	6.3	0.17	21
								Mean 22

T = turnover time. T/2 = half life.

the same direction. Thus, even though it is possible that the concentrations of NEFA found were erroneously high, it is probable that the difference between the concentration of NEFA in adipose tissue of glucose fed and starving rats is correct. This difference may be regarded as a further support for the accepted concept that transport of fat from fat depots occurs in the form of NEFA.

Elimination and recycling of NEFA in glucose fed rats. Complete interpretation of the complicated course of the elimination of palmitic acid from the plasma and its recycling in the latter as TG (Fig. 1—2) is not possible on the basis of our present knowledge. In an attempt to facilitate the understanding of these curves, it might be convenient first to dwell on the results from experiments of the turnover of TG in glucose fed rats (see Fig. 6).

The curves for the specific activity of NEFA in these experiments are complicated by the fact that up to 30 per cent of the activity in the serum used for injection was localised to NEFA. From experiment 1 (Fig. 1), however, it can be calculated that already after 6 min only about 1 per cent of the palmitic acid injected in non-esterified form was found in the plasma. This corresponds to about 5 per cent of the activity found in NEFA after 6 min in the glyceride turnover experiment no. 5 and 6. The rest of the activity in NEFA at that time must have come from plasma TG after hydrolysis of the latter. This thus shows that a fair portion of the fatty acids in the endogenous plasma TG are rapidly recycled in the plasma as NEFA before they leave the blood stream, an observation analogous to what HAVEL and FREDRICKSON (1956) found for triglycerides in chylomicrons.

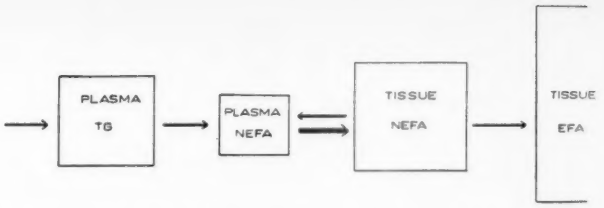

Fig. 3. The simplest model fitting the experimental results in glucose fed rats. EFA = esterified fatty acids.

The curves for the elimination and recycling of glucose fed rats (Fig. 1 and 2) show that the specific activity of plasma NEFA and TG were almost identical after the activity of TG in the plasma had reached a peak value. This would have occurred if all NEFA in plasma under these conditions emanated from plasma TG fatty acids. Judging from a previous study of the distribution of palmitic acid (LAURELL 1959), no other source of plasma NEFA is evident under these conditions.

It is apparent from Table II, however, that the turnover rate of NEFA in glucose fed rats was found to be about three times that of the plasma TG. This turnover rate was, however, calculated, as mentioned, so that it included not only the net transport of fatty acids between different tissues through plasma but also the exchange of NEFA, which probably occurs between plasma and tissue pools without net transport. The values for the NEFA turnover in Table II may also have been increased erroneously if TG in the analysed plasma samples had to any extent undergone hydrolysis *in vitro*, which would have resulted in over-estimation of the plasma NEFA pool without influencing the specific activity ratios. With these reservations it appears probable that most of the plasma NEFA in glucose fed rats is derived from hydrolysis of plasma TG. The question as to what extent the endogenous plasma TG is recycled as NEFA, on the other hand, requires more experimental data for analysis analogous with the chylomicron transport (FREDRICKSON 1958). The simplest system fitting the experimental data is given in Fig. 3.

After administration of C^{14} labelled acetate to dogs HARPER, NEAL and HLAVACEK (1953) found a course of the activity of non-phospholipid fatty acids in the plasma that agrees essentially with the present results after administration of palmitic acid-1-C^{14}. By series of liver biopsy and hepatectomy these authors were able to show that, practically speaking, all of the activity in the plasma fatty acids must have come from lipids synthetised in the liver. In Fig. 1 and 2 the specific activity in the liver TG is marked by a value, determined at the end of the experiment as well as by a value at 20 min, which is based on an earlier observation (LAURELL 1959) that about 24 per cent of the injected activity at that time was in the liver TG. Fig. 1 and 2 make it probable

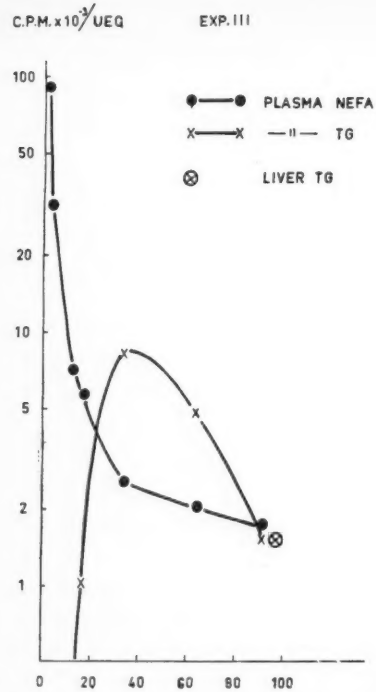

Fig. 4. Disappearance and recycling of injected palmitic acid-1-C^{14}. Starving rats. Horizontal axis: min after the injection.

that the specific activity of the liver TG falls parallel to that of the plasma TG. The values however, are hardly compatible with a simple precursor-product course. A number of pools of partly unknown size must also be passed by the palmitic acid before the latter leaves the liver in esterified form probably built into lipoproteins. The results support the assumption that the recycling of plasma NEFA as plasma TG occurs mainly via the liver TG during glucose administration, but charting of the course requires further experimental data.

Recycling of NEFA in starving rats. The curves obtained in the experiments on the recycling of palmitic acid in plasma TG in starving rats (Fig. 4 and 5) differed from the curves for glucose fed rats in various respects. The specific activity of the plasma NEFA in the experiments with starving rats was less than half of that found for plasma TG at the time of the peak activity (40 min) of the latter and afterwards approached the specific activity of the plasma TG more and more. The wide difference in specific activity for the 40 min values may be explained by the fact that plasma NEFA in starving rats must in the main be supposed to come from adipose tissues with low specific activity (Fredrickson and Gordon 1958 a). However, that even in these experiments part of the NEFA is derived from plasma TG is obvious from Fig. 7. The fact

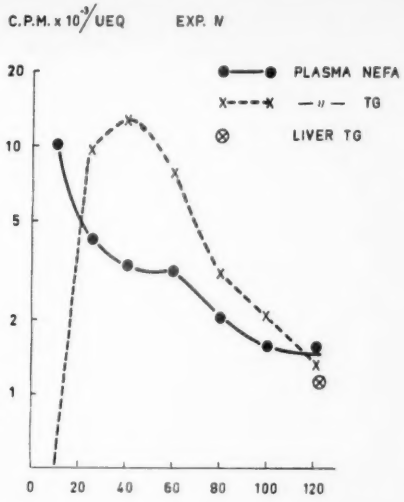

Fig. 5. Disappearance and recycling of injected palmitic acid-1-C^{14}. Starving rats. Horizontal axis: min after the injection.

that the specific activity of NEFA and TG nevertheless approach one another is probably due to the rapid fall in the activity of the plasma TG and partly to the time lag of the activity of NEFA due to exchange between plasma NEFA and pools in the tissues, surely represented by NEFA in adipose tissue.

At the end of these experiments the specific activity of the liver TG was very close to that of the plasma TG in contradistinction to what was seen in glucose fed rats. This result might be expected if the pools in the liver, which the palmitic acid must pass on its way from the plasma NEFA to the plasma TG, is passed more quickly in starving rats than in glucose fed rats. This explanation is, however, less likely because it is apparent from the results that the peak activity of the plasma TG occurred at roughly the same time in all experiments. An alternative explanation might be that a considerable part of the plasma TG in starving rats was derived from a source of lower specific activity than that of the liver TG. This possibility cannot be excluded without more detailed investigation of the ratio between the specific activity of the liver and of the plasma TG. In a previous investigation (LAURELL 1959) between 8.6 and 16.7 per cent of the palmitic acid-1-C^{14} was recovered from the liver TG 20 min after injection of the isotope into starving rats. This would correspond to an upper limit of the specific activity in liver TG after 20 min of 11,000 cpm/μeq in exp. 3 and 13,000 cpm/μeq in exp. 4. Both these values are more than enough to fit a precursor product relationship between liver TG and plasma TG.

Another mechanism that must influence the specific activity of the liver TG in relation to the plasma TG is the difference in fat metabolism in the liver of starving and glucose fed rats. As pointed out above, the net transport of NEFA in plasma is probably at most as large as that of plasma TG in glucose fed rats.

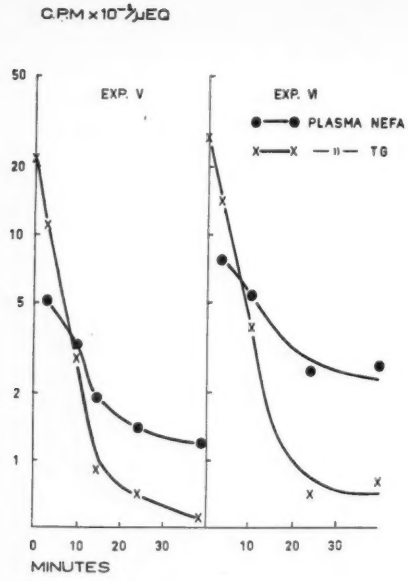

Fig. 6. Disappearance of injected labelled plasma TG and their recycling as NEFA. Glucose fed rats.

About 25 per cent of this transport goes to the liver TG. Simultaneously there is a continuous new-synthesis of fatty acids from carbohydrates via acetyl-CoA. In a steady state, then, at least 75 per cent and possibly more of the pool fatty acyl-CoA in the liver must be derived from unlabelled fatty acids. These fatty acids are precursors of liver TG. In starving rats, on the other hand, new-synthesis of fatty acids from carbohydrates is negligible (LYON, MASRI and CHAIKOFF 1952) which results in fatty acyl-CoA in the liver of these animals probably having a specific activity of the same order as plasma NEFA and thus much higher than in glucose fed rats. The specific activity of the liver TG falls rapidly in starving rats in these experiments, which may in part be due to losses in the form of $C^{14}O_2$, and is reflected in the specific activity of the plasma TG in exp. 3 and 4. This rapid fall together with the time lag in the transport system plasma NEFA — liver TG — plasma TG — plasma NEFA may be expected to result in fluctuations in the ratios between the specific activities in the pools of the system and might explain the similarity found in specific activity of the liver and plasma TG at the end of the experiments.

Recycling of NEFA as phospholipids. The specific activity of the plasma phospholipids in all of the experiments showed the same course as that described earlier in experiments with glucose fed rats (LAURELL 1959). Not even in any of the experiments that were continued for 2 hours was any peak found and the specific activity of the phospholipids was less than that of the plasma TG throughout. To what extent the exchange between the liver phosholipid

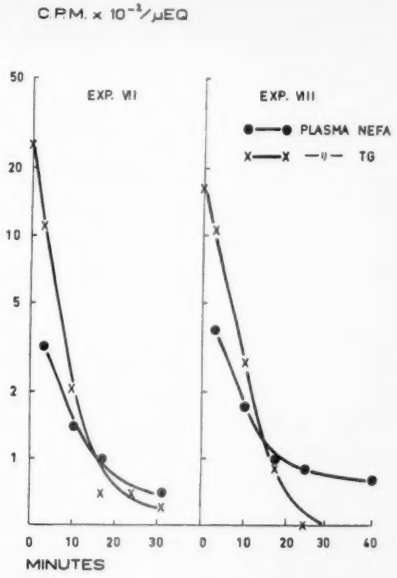

Fig. 7. Disappearance of injected labelled plasma TG and their recycling as NEFA. Starving rats.

fatty acids and liver TG fatty acids occurs is unknown. It has been shown previously that the recovery of injected palmitic acid in liver phospholipids is 15—19 per cent in glucose fed rats and 9—14 per cent in starving rats (LAURELL 1959). There is thus an established connection between the liver pools of TG and phospholipids via the plasma TG and NEFA. Since no appreciable net transport of phospholipids from the liver occurs (ENTENMAN, CHAIKOFF and ZILVERSMIT 1946), a steady state — at least in glucose fed rats — appears to require transport of phospholipid fatty acid to the TG-pool in the liver without passage of plasma. These two ways alone between the pool under discussion in the liver must lead to further complications in the course of the elimination curves.

Turnover rate of plasma TG. In Fig. 6 and 7 typical curves are given for the specific activity of the plasma TG and NEFA in experiments carried out to determine the turnover rate of plasma TG by injection of serum containing plasma TG with high specific activity. It is clear that the elimination curve for the plasma TG was exponential until about 95 per cent had been eliminated. The slower phase that then followed was partly, and towards the end probably entirely, due to recycling of labelled fatty acids taken up earlier by the liver. In exp. 6 the specific activity of the liver TG at the end of the experiment was thus twice as high as that of the plasma TG at the same time (180 cpm/μeq).

The absence of a peak in the specific activity of NEFA in these experiments is due to the long intervals between collection of the samples. In two experiments

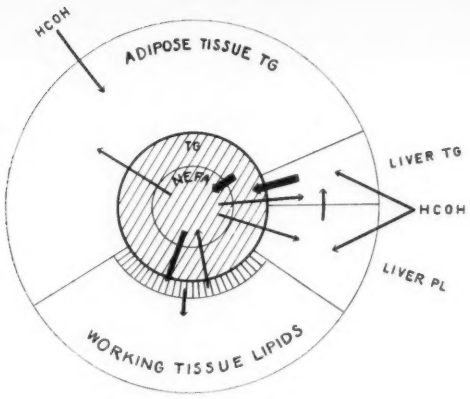

Fig. 8. Symbolic representation of the transport of endogenous fatty acids in plasma fitting available experimental evidence. Glucose fed rats. Hatched areas: (a) diagonal, denotes plasma pools, (b) radially, denotes tissue NEFA pool. HCOH = carbohydrate. PL = phospholipids.

on a glucose fed and a starving rat, in which samples were drawn at short intervals initially, a minimum was noted in the specific activity of the plasma NEFA when about 50 per cent of the TG activity of the plasma had been eliminated and a maximum when 75—90 per cent had been eliminated. Since this complicated course was studied only in single experiments, they will not be discussed further. However, both these results and the high specific activity of plasma NEFA at the end of the other experiments suggest that a considerable part of the plasma TG fatty acids may be recycled as plasma NEFA after intact plasma TG has left the circulation.

The recovery of the activity in the liver TG and phospholipids after these experiments was determined in two glucose fed and two starving rats 15 and 40 min after injection of serum containing radioactive TG and NEFA. After 15 min less activity was recovered and after 40 min more than what was observed 20 min after injection of palmitic acid-1-C^{14}. Discussion of the distribution of the plasma TG pattern must, however, abide further experimental data.

Table 2 gives the calculation of the turnover rate of the plasma TG based on these results. The table also includes approximate values for the turnover rates of NEFA in the same experiments based on the turnover times, that had been determined previously for this fraction. The results in Table 2 obtained in the experiments on glucose fed rats have already been discussed and resulted in certain difficulties in interpretation. In the experiments on starving rats the turnover of the plasma TG was, on the average, 22 per cent of that of the plasma NEFA. It has been shown previously that 8.6—16.7 per cent of the injected palmitic acid-1-C^{14} was recovered 20 min later in the liver TG. These results need not be incompatible with the concept of recycling of fatty acids during

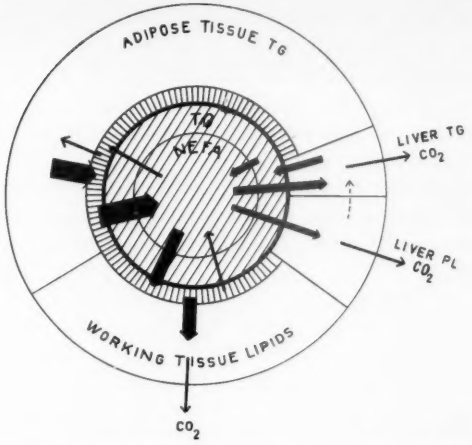

Fig. 9. Symbolic representation of the transport of endogenous fatty acids in plasma fitting available experimental evidence. Starving rats. For explanation see Fig. 8.

starvation described in the present paper but it is obvious that much more experimental data are necessary to form anything like a correct opinion of the amounts transported.

The present investigation together with earlier known facts suggest a picture of the transport of fatty acids in plasma as endogenous TG and NEFA, which may be summarised in the mamillary systems given in Fig. 8 and 9. As a rule the width of the arrows in these figures is intended to indicate semiquantitatively the importance of the different transport routes. The exceptions are the parts of the arrows corresponding to exchange of NEFA between plasma and tissues and the arrows indicating formation of fatty acids from carbohydrates and CO_2 from fatty acids. The NEFA-pool in "working tissue" is hypothetical and the absence of such pools in adipose tissue in Fig. 9 and in liver only means that they appear to be comparatively small.

Some essential differences between the systems which correspond to glucose feeding and starvation, respectively, will be dwelt on further below. In glucose fed rats the plasma NEFA pool are smaller than in starving rats and derived mainly from plasma TG. Oxidation of fatty acids (Mc Calla, Gates and Gordon 1957) and probably also the outflux of keton bodies from the liver are small, as is the outflux of fatty acids from adipose tissue. An active synthesis of fatty acids from carbohydrates on the other hand, occurs under these conditions, especially in the liver and adipose tissue (Feller 1954). The net transport occurring during glucose feeding implies transport of fatty acids newly synthetised in the liver to working and adipose tissues.

In starving rats the plasma NEFA pool is larger and the most important

inflow consists of fatty acids from adipose tissues, although some is derived from plasma TG. The fatty acids arived to the liver are oxidized partly to CO_2 and retransported partly as plasma TG and partly as keton bodies. New-synthesis of fatty acids from carbohydrates is small (LYON, MASRI and CHAIKOFF 1952). The resulting net transport during starvation is thus directed from adipose tissues to working tissues.

The quantitative pattern in this complex transport system is still vague and a number of alternative transport passages are also possible. This applies in particular to plasma TG whose elimination other than via plasma NEFA has not been investigated.

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The Release of Catechols from the Adrenal Medulla on Activation of the Bulbar Part of the Sympathetic Vasodilator Outflow in Cats

By

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Abstract

LINDGREN, P., A. ROSÉN and B. UVNÄS. The release of catechols from the adrenal medulla on activation of the bulbar part of the sympathetic vasodilator outflow in cats. *Acta physiol. scand.* 1959. *47.* 233—242. — In a previous investigation it was observed that activation of the sympathetic vasodilator outflow by hypothalamic stimulation was accompanied by a discharge of catechols from the adrenals, consisting mainly of adrenaline. The present experiments were undertaken in order to study the adrenal function during medullary stimulation of this pathway. The catechol output from one adrenal gland was observed to increase on activation of the sympathetic vasodilator outflow by bulbar stimulation. Adrenaline increased relatively more than noradrenaline. The mean values for the adrenaline output in the control samples and those taken during the stimulation periods were 0.05 and 0.22 μg/kg/min (increase 340 per cent). The corresponding figures for noradrenaline were 0.22 and 0.50 μg/kg/min (increase 130 per cent). The findings are in accordance with our previous results showing that intracerebral stimulation of the sympathetic vasodilator outflow is accompanied by a discharge of catechols — predominantly adrenaline — from the adrenals. The amounts secreted are of such a low magnitude as to have only minor vascular effects.

As repeatedly reported from our laboratory the sympathetic vasodilator outflow in cats and dogs seems to be confined to the skeletal muscles. Activation of the outflow by intracerebral stimulation at various levels produces vasodilator responses only in the muscles. Concomitant vasoconstrictions in the cutaneous and splanchnic areas indicate that vasoconstrictor neurons accompanying the vasodilator neurons are activated by the same stimuli. The simultaneous activation of vasodilators to muscle vessels and vasoconstrictors to skin and abdominal vessels has led us to speculation concerning the functional significance of the vasodilator outflow (for details see UVNÄS 1954).

A previous paper (GRANT et al. 1958) reported the observation that activation of the sympathetic vasodilator outflow by hypothalamic stimulation was accompanied by a discharge of catechols from the adrenals. The discharge consisted mainly of adrenaline.

LINDGREN (1955) observed that activation of the mesencephalic part of the sympathetic vasodilator outflow also resulted in a discharge of catechols. The ratio of adrenaline to noradrenaline was not determined, but the proportion of adrenaline was believed to be at least 25 per cent since vasodilatation occurred in denervated skeletal muscles. According to LINDGREN's own experience a mixture of adrenaline and noradrenaline given intravenously induces a vasodilatation when the adrenaline content of the mixture amounts to 25 per cent or more.

From these previous investigations we were uncertain whether the increase of the catechol output observed constituted part of a reaction pattern characteristic of sympathetic vasodilator activation or if this increase was due to the spread of the stimuli to adjacent structures without any functional relationship to the vasodilator fibres.

In the oblongate medulla the sympathetic vasodilator pathway — with accompanying vasoconstrictor fibres — passes as a rather well defined bundle in the ventrolateral area (LINDGREN et al. 1956). As this area is apart from regions which are supposed to govern the medullary vasomotor control (vasoconstrictor centre, depressor area, etc.) conditions are favourable for a selective activation of the vasodilator outflow and the accompanying fibres at this level. The adrenal output of catechols was therefore determined during activation of the bulbar vasodilator outflow.

Methods

The material was comprised of 6 cats. The animals were anaesthetized with intravenous injections of urethane (800—1,200 mg/kg) in five experiments and with Dial (50 mg/kg) in one.

The occipital bone and the atlanto-occipital membrane were removed and topical stimulation in the medulla was applied with either a unipolar or a bipolar electrode oriented by means of a Horsley-Clarke apparatus (for details see LINDGREN and UVNÄS

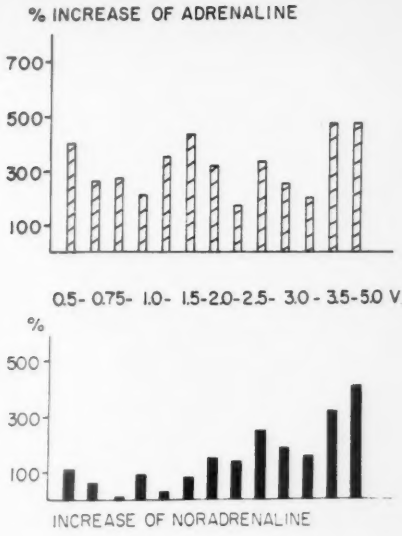

Fig. 1. Catechol output from one adrenal gland on activation of the bulbar part of the sympathetic vasodilator outflow. Relative values from 13 stimulations. Survey of the results from 6 experiments on cats anaesthetized with urethane or (in one cat) Dial. Percentual increase is calculated from the control level taken 5 min before each stimulation.

1953). The electric stimuli used were rectangular voltage pulses; their duration was 2 msec, their voltage 0.5—5 V and their frequency 70 per sec.

For recording the blood flow from a skeletal muscle region, a hind leg was skinned and the femoral vein was cannulated and the blood directed to a phototube drop-counter operating an ordinate recorder (LINDGREN and UVNÄS 1954). To prevent clotting, heparin was given i.v. (25 mg/kg).

The blood pressure was measured in a carotid artery with a mercury manometer.

The brains were examined histologically for localizing the electrode punctures and stimulation points.

Blood samples from the left adrenal vein were collected during control and stimulation periods, and were tested (rat uterus and rat colon) after separating adrenaline and noradrenaline by paper chromatography. The catechol assay was performed as described in a previous paper (GRANT *et al.* 1958).

Results

The catechol discharge was studied in 13 stimulations of *the bulbar part of the sympathetic vasodilator outflow*. In all experiments an increase of the adrenaline discharge was observed. The percentage change of catechols in every stimulation is seen in Fig. 1. As shown in Fig. 2, the mean values for the adrenaline output in the control samples and those taken during stimulation were 0.05 and 0.22 μg/kg/min respectively. The corresponding figures for noradrenaline were 0.22 and 0.50 μg/kg/min. The maximal output of the adrenaline during stimulation amounted to 0.51 μg/kg/min, that of noradrenaline to 0.75 μg/kg/

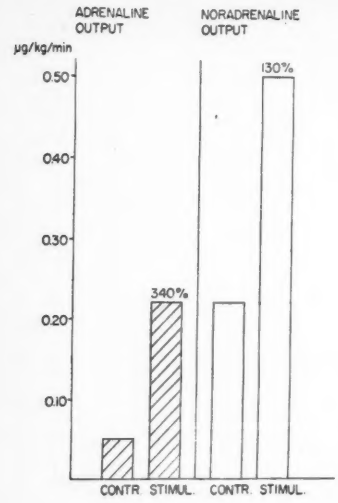

Fig. 2. Mean values for the adrenaline and noradrenaline output due to stimulation in the oblongate medulla (13 control and 13 stimulation periods).

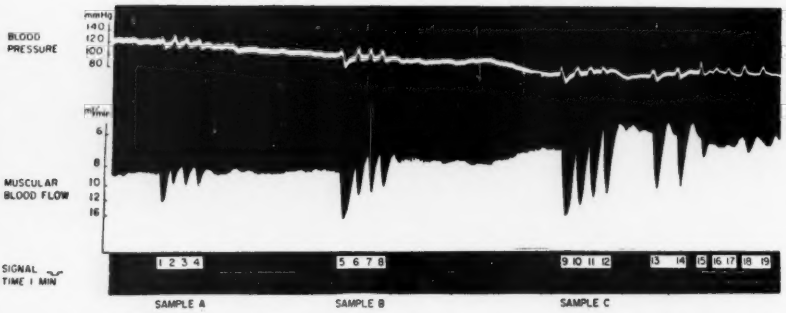

Fig. 3. Cat no. 3. 3.5 kg. Urethane 1,150 mg/kg. Vasomotor response in the muscles of the right hind leg to stimulation of the sympathetic vasodilator pathway in the oblongate medulla. All stimulations applied to the same point with 70 imp/sec. Samples A, B and C indicate the catechol output seen in Fig. 4.

1, 2, 3, 4 Stimulation, 2.5 V.
5, 6, 7, 8 Stimulation, 3.5 V.
9, 10, 11, 12 Stimulation, 5.0 V.
13, 14 Stimulation, 3.5 V.
15 Atropine. 0.2 mg/kg i.v.
16, 17 =13, 14.
18, 19 Stimulation, 5.0 V.

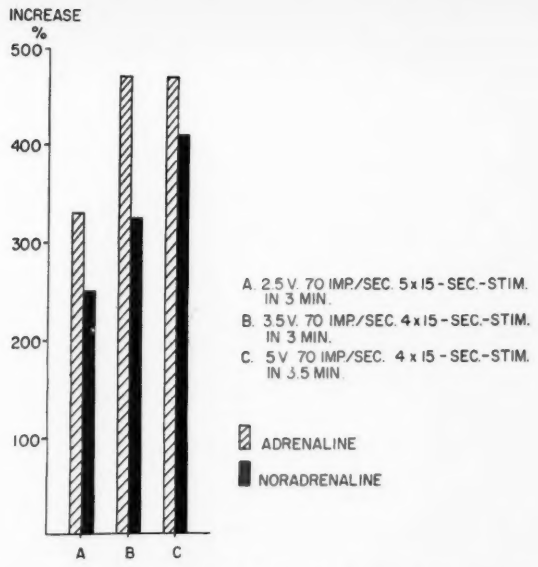

Fig. 4. Catechol output from the left adrenal gland on activation of the sympathetic vasodilator outflow by stimulation in the oblongate medulla (the same experiment as illustrated in Fig. 3). The percentual increase is calculated from the control level recorded 5 min before each stimulation.

min. The adrenaline content of the adrenal blood increased by an average of 340 per cent. The noradrenaline also increased in most experiments, although to a lesser degree, averaging 130 per cent. As a control test of the electrode position the vasomotor response in the skeletal muscles to bulbar stimulation was recorded in all experiments. Only vasodilator effects that were blocked by low doses of atropine were attributed to vasodilator activity.

An experiment is illustrated in Fig. 3. A bulbar vasodilator point was stimulated, and the discharge of catechols from the left adrenal gland was determined during three different stimulation periods. The intensity of the stimuli was equivalent to 2.5, 3.5 and 5.0 V (A, B and C in Fig. 3). At 13 and 14 two additional stimulations were given with an intensity corresponding to 3.5 V. Atropine was then given in a dose of 0.2 mg/kg intravenously. Repeated stimulations with 3.5 V (at 16 and 17) and 5.0 V (at 18 and 19) now failed to produce any vasodilatation. In other words, the vasodilatation was considered to have been produced by vasodilator discharges. Histologic examination showed, in agreement with this interpretation, that the point of stimulation was situated in the region where, in previous investigations, the sympathetic vasodilator outflow has been observed to pass (LINDGREN et al. 1956).

Table 1. Catechol output from one adrenal gland on bulbar stimulation

	Weight	Anaesthesia	Electrode	Adrenaline µg/kg/min	Noradrenaline µg/kg/min	% Adrenaline	% Adrenaline increase	% Noradrenaline increase
Cat no. 1:	3.3 kg	Dial	bipolar	—	—	—	—	—
Control	—	—	—	0.02	0.23	8	—	—
Stimulation 3 V	—	—	—	0.07	0.66	10	250	187
Stimulation 3 V (same point)	—	—	—	0.06	0.61	9	200	165
Cat no. 2:	3.0 kg	Urethane	bipolar	—	—	—	—	—
Control	—	—	—	0.03	0.14	18	—	—
Stimulation 2.5 V....	—	—	—	0.08	0.33	20	167	136
Control	—	—	—	0.03	0.17	15	—	—
Cat no. 3:	3.5 kg	Urethane	bipolar	—	—	—	—	—
Control	—	—	—	0.06	0.08	43	—	—
Stimulation 2.5 V....	—	—	—	0.26	0.28	48	330	250
Stimulation 3.5 V (same point)	—	—	—	0.34	0.34	50	470	325
Control	—	—	—	0.09	0.14	39	—	—
Stimulation 5 V (same point)	—	—	—	0.51	0.72	42	470	410
Control	—	—	—	0.11	0.29	27	—	—
Cat no. 4:	3.5 kg	Urethane	unipolar	—	—	—	—	—
Control	—	—	—	0.03	0.29	9	—	—
Stimulation 1.5 V....	—	—	—	0.16	0.49	25	430	69
Control	—	—	—	0.03	0.33	8	—	—
Cat no. 5:	3.5 kg	Urethane	unipolar	—	—	—	—	—
Control	—	—	—	0.07	0.17	29	—	—
Stimulation 2 V	—	—	—	0.29	0.43	40	315	153
Control	—	—	—	0.04	0.19	17	—	—
Stimulation 1 V (same point)	—	—	—	0.18	0.24	43	350	26
Control	—	—	—	0.03	0.20	13	—	—
Stimulation 0.75 V (same point)	—	—	—	0.11	0.21	34	270	5

	Weight	Anaesthesia	Electrode	Adrenaline µg/kg/min	Noradrenaline µg/kg/min	% Adrena line	% Adrenaline increase	% Noradrenaline increase
Cat no. 6:	3.5 kg	Urethane	unipolar	—	—	—	—	—
Control	—	—	—	0.08	0.39	17	—	—
Stimulation 1 V	—	—	—	0.25	0.75	25	210	92
Control	—	—	—	0.07	0.48	13	—	—
Stimulation 0.75 V (same point)	—	—	—	0.25	0.75	25	260	56
Control	—	—	—	0.05	0.38	12	—	—
Stimulation 0 50 V (same point)	—	—	—	0.25	0.75	25	400	97
Control	—	—	—	0.05	0.28	16	—	—

Fig. 4 gives the catechol figures obtained during stimulation periods A, B and C. In all of them we observed a significant rise of the catechol output with some preponderance for the discharge of adrenaline, which increased up to 470 per cent, while the noradrenaline increase was maximally 410 per cent.

The plasma level for adrenaline and noradrenaline was within the same range as that in previous experiments conducted by GRANT *et al.* The initial values were 0.02—0.08 µg/kg/min for adrenaline and 0.08—0.39 µg/kg/min for noradrenaline. The plasma levels of the catechols, as observed by GRANT et al., slowly increased during the course of the experiments.

The data from all the experiments are detailed in Table I.

Localization. The localization of five stimulated points observed at histologic examination is shown in Fig. 5. All vasodilator points are concentrated to the ventrolateral part of the oblongate medulla just dorsolateral to the pyramids.

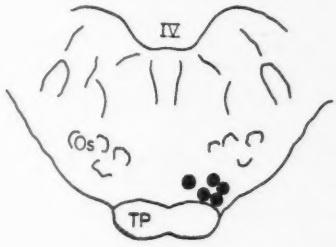

Fig. 5. Drawing of a frontal section through the oblongate medulla of a cat's brain. Stimulation points, histologically localized to a region 1.5 mm anterior or posterior to this section, are represented by the black circles.

Os Superior olive
TP Pyramidal tract
IV Fourth ventricle

Discussion

The activation of the sympathetic vasodilator outflow at its medullary level is accompanied by a discharge of catechols from the adrenals. The physiological significance of this observation is obscure, but the fact that a similar discharge of catechols occurs on the activation of the sympathetic vasodilator outflow at hypothalamic or mesencephalic levels might indicate that a catechol discharge constitutes part of a reaction pattern characteristic of the activation of the vasodilator outflow.

Previously LINDGREN and UVNÄS (1953) found that the vasodilator neurons are accompanied by vasoconstrictor neurons to the skin and abdominal regions. We do not know under what circumstances these vasodilator, vasoconstrictor and adrenomotor neurons are activated. In earlier papers we propounded the hypothesis that the sympathetic vasodilator outflow constitutes an integrative part of an emergency effector system since the concomitant activation of vasodilator fibres to the muscles and vasoconstrictor fibres to the cutaneous and splanchnic areas produces a shift of the circulating blood from inactive to active areas. A concomitant discharge of catechols should promote such a redistribution of the peripheral blood circulation, since at the adrenaline/noradrenaline ratio observed a catechol output, if high enough, dilates the muscle vessels and constricts the cutaneous and splanchnic vessels.

On activation of the sympathetic vasodilator outflow by hypothalamic stimulation GRANT *et al.* observed a discharge from the adrenal medulla consisting mainly of adrenaline. The ratio of adrenaline to noradrenaline (A/N ratio) changes from 24/76 to 48/52, a change which, with the low initial adrenaline values observed, means a substantial increase of the adrenaline discharge (mean increase 433 per cent) but a much lower one for noradrenaline (mean increase 84 per cent). On mesencephalic stimulation LINDGREN (1955) found a catechol output containing at least 25 per cent adrenaline — sufficient to grant the catechol mixture vasodilator action on the muscle vessels. In the present experiments, too, the adrenaline rise was dominant though less markedly so. The A/N ratio changed from 19/81 to 31/69. The quantitative differences observed in the total output of catechols as well as in the A/N ratio on activation of the sympathetic vasodilator outflow at various brain levels are worth noting but might be without any physiological significance since the differences might have an experimental background (different spread of stimuli, anatomic differences in the distribution of the various neurons, different thresholds, etc.) at the various levels of stimulation.

Medullary vasomotor reflexes have been observed to be attended by the discharge of catechols from the adrenals. TOURNADE and CHABROL (1926), and HEYMANS (1929) found that carotid sinus reflexes influenced the output of the medullary hormones. Occlusion of the carotid arteries, according to HOLTZ and SCHÜMANN (1949), and BRAUNER *et al.* (1950), causes a discharge

mainly of adrenaline. On the other hand, KAINDL and EULER (1951) reported no change in the A/N ratio in the adrenal discharge under such circumstances despite a fourfold increase in the total output.

It is less probable that the reflex output of catechols observed by the aforementioned authors is mediated by fibres in the ventrolateral sympathetic outflow described by us in previous papers, since experimental evidence so far obtained indicates that this outflow — at least as far as the vasodilator fibres are concerned — does not take part in medullary reflexes.

A quantitative comparison of our observations with earlier ones concerning the catechol discharge on bulbar stimulation and in medullary vasomotor reflexes is not very rewarding. In several previous papers no distinction has been made between adrenaline and noradrenaline and, in recent experiments in which this has been done, the results are rather varying (see above).

In experiments with intravenous infusion of adrenaline and noradrenaline respectively in cats LINDGREN, ROSÉN and UVNÄS (1959) found that an infusion rate below 0.25—1.0 μg/kg/min adrenaline was too low to evoke vascular responses in different organs. Comparison of this figure with the results of the present investigation shows that the catechol outflow during the periods of stimulation was only exceptionally of a magnitude such that it could to any great degree affect the peripheral circulation.

The nervous component (vasodilator fibres to the musculature, vasoconstrictor fibres to skin and intestines) would therefore seem to play the dominant role in the redistribution of blood that results from stimulation of the vasodilator pathway, regardless of whether that activation takes place from the hypothalamus, mesencephalon or oblongate medulla. It has also been observed earlier, however, that the humoral component (adrenal activation) can play a supporting role in isolated experiments (LINDGREN 1955, GRANT *et al.* 1958).

The expenses of this investigation were partly defrayed by a grant from the Swedish Medical Research Council, which is gratefully acknowledged.

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Quantitative Aspects of the Vasomotor Action of Adrenaline and Noradrenaline in the Cat. Relationship to Catechol Discharge on Intracerebral Stimulation of the Sympathetic Vasodilator Outflow

By

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Abstract

LINDGREN, P., A. ROSÉN and B. UVNÄS. Quantitative aspects of the vasomotor action of adrenaline and noradrenaline in the cat. Relationship to catechol discharge on intracerebral stimulation of the sympathetic vasodilator outflow. *Acta physiol. scand.* 1959. *47*. 243—250. — In order to study to what extent the catechol output from the adrenals during central activation of the sympathetic vasodilator pathway might contribute to the vascular reactions, the vasomotor effects of adrenaline and noradrenaline infusions were investigated. Intravenous infusion of adrenaline in doses above 0.25—2 μg/kg/min produced vasodilatation in the skeletal muscles in cats under Dial or urethane anaesthesia. Noradrenaline caused vasoconstriction in doses of 1 μg/kg/min and more. Hypothalamic or medullary activation of the sympathetic vasodilator outflow might produce a discharge of the two catechols sufficient to reach these threshold levels if care is not taken to avoid stimulation with high intensity.

The discharge of adrenaline from the adrenals has long been thought to play an important role in the control of the peripheral blood flow, at least under stress conditions such as cold environment, muscular exercise, emergency

situations etc. Lately a noradrenaline discharge has also been ascribed an important function. Recent observations by CELANDER (1954) are rather contradictory to such an opinion. He contended on the basis of experiments on anaesthetized cats that the catechol discharge from the adrenals was too low to influence the vasomotor tone significantly.

During the course of our own experiments concerning the sympathetic vasodilator outflow to the skeletal muscles it was observed that the vasodilator activation brought about by intracerebral stimulation was accompanied by a discharge of catechols from the adrenals (GRANT *et al.* 1958, LINDGREN, ROSÉN and UVNÄS 1959). The question arose to what extent this catechol output might contribute to the vascular reaction pattern obtained. We therefore decided to study quantitatively the action of the two adrenal catechols on the peripheral blood flow especially in the muscles.

Method

The experiments were performed on 12 cats under Dial (40—60 mg/kg) or urethane (800—1,500 mg/kg) anaesthesia. Solutions of adrenaline and noradrenaline were infused intravenously. Blood flow was measured with the photoelectric drop recording technique previously described (LINDGREN and UVNÄS 1954). Recordings were made of the flow in a muscle region (v. femoralis in a skinned leg), a skin region (v. saphena magna) and in the splanchnic area (a branch of the v. mesenterica superior). The blood pressure was measured in the carotid artery. Heparin (25 mg/kg) in 5 per cent solution was given intravenously at the beginning of the experiment.

Results

Adrenaline in small doses produced an increase of muscle blood flow due to vasodilatation as indicated by the decrease in peripheral resistance. The vasodilator threshold dose for adrenaline seemed to be a little smaller in cats anaesthetized with Dial, 0.25—1.0 μg/kg/min, than for urethane anaesthesia, 1.0—2.0 μg/kg/min. The threshold dose giving a rise in the blood pressure was 1.0—2.0 μg/kg/min in cats anesthetized with Dial. With increasing adrenaline concentrations the vasodilator action was more and more counteracted by a vasoconstrictor component, causing a successive increase in the peripheral resistance (Fig. 4). In spite of the vasoconstriction the muscle blood flow still increased due to the concomitant rise in blood pressure.

A typical experiment is shown in Fig. 1. Increasing amounts of adrenaline were infused, starting with a subthreshold dose, 0.17 μg/kg/min. In this case the vasodilator threshold seemed to be 0.30 μg/kg/min. The small doses, below 0.5 μg/kg/min, did not influence the blood pressure; the systemic effect of the vasodilator response in the muscles had apparently been counteracted by vasoconstrictor responses in other tissues. When larger doses of adrenaline were infused, the responses were dominated by the blood pressure rise. In

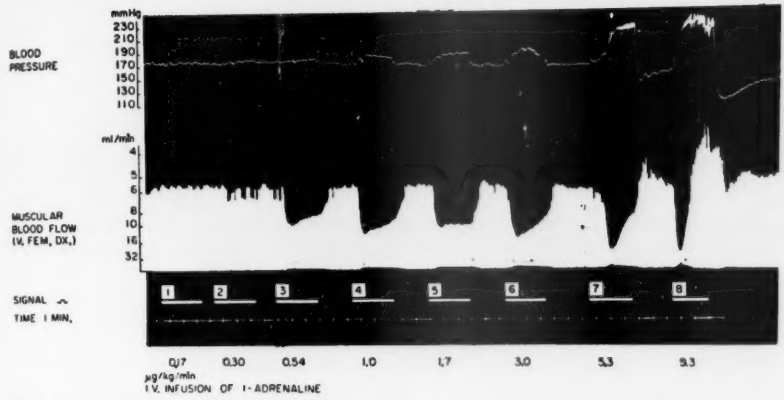

Fig. 1. Cat 2.8 kg. Dial 50 mg/kg.
Vascular responses in the muscles of the right hind leg to intravenous infusion of increasing amounts of adrenaline.

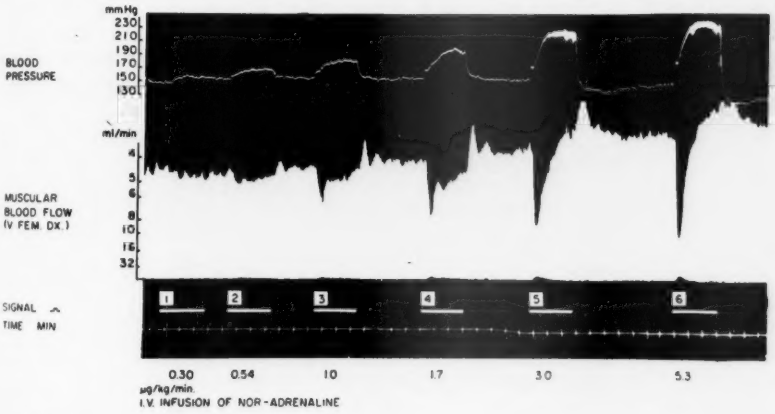

Fig. 2. The same cat as in Fig. 1.
Vascular responses in the muscles of the right hind leg to intravenous infusion of increasing amounts of noradrenaline.

order to get a more illustrative picture of the changes in the peripheral resistance we have calculated this factor in the manner described by GREEN *et al.* (1944). As seen from Fig. 3, the maximal vasodilator activity of adrenaline was exerted in this experiment when a dose of 1—3 µg/kg/min was infused. Fig. 4 shows similar dose response curves from 4 experiments.

In contrast to adrenaline, noradrenaline excited pure vasoconstrictor action,

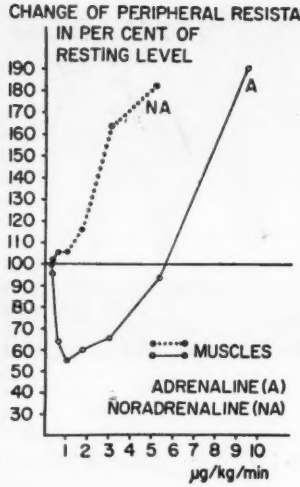

Fig. 3. Changes in peripheral resistance in the muscles during the experiments shown in Fig. 1 and 2.
Peripheral resistance (PR) is calculated according to GREEN, LEWIS, NICKERSSON, HELLER (1944).

$$\left(\text{PR-Unit} = \frac{1 \text{ mm Hg}}{1 \text{ ml/min}}\right)$$

In the diagram the change in PR-Units is given in percentage of resting level.

the peripheral resistance showing a sharp rise as soon as the threshold dose was passed (Fig. 3). The threshold dose giving blood pressure effects was 0.25—1.0 µg/kg/min. The increase in blood flow observed during noradrenaline infusion was evidently secondary to the vasopressor effects. Typical responses

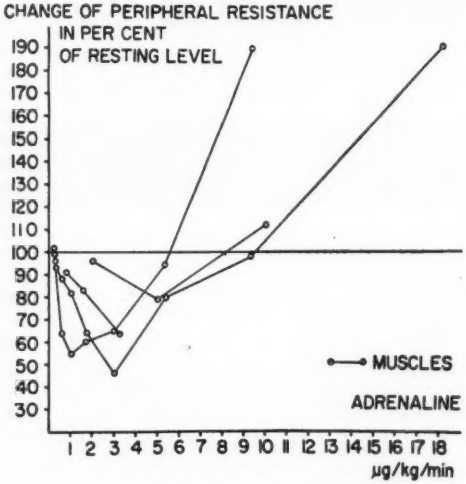

Fig. 4. Changes in peripheral resistance in the skeletal muscles of a hind leg in four cats anaesthetized with Dial, during intravenous infusion of increasing amounts of adrenaline.

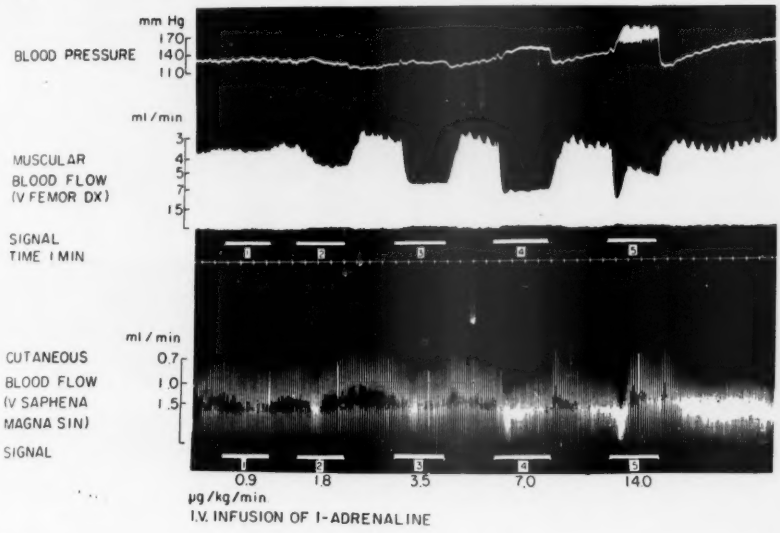

Fig. 5. Cat 2.7 kg. Urethane 1,500 mg/kg.
Vascular responses in the muscles of the right hind leg and in the skin of the left paw to intravenous infusion of increasing amounts of adrenaline.

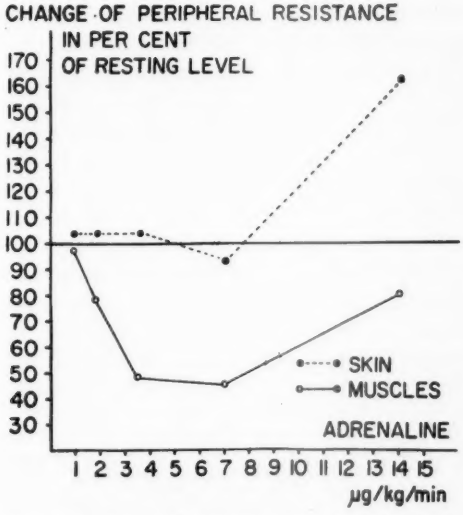

Fig. 6. Changes in peripheral resistance in the experiment shown in Fig. 5.

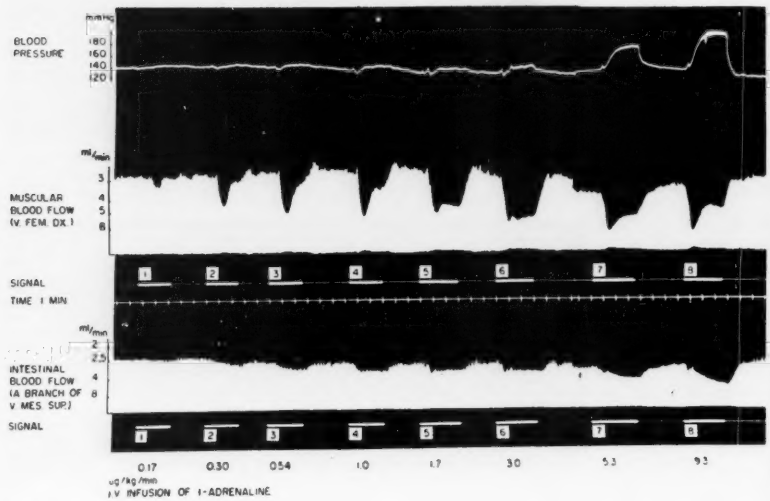

Fig. 7. Cat 2.7 kg. Dial 55 mg/kg.
Vascular responses in the muscles of the right hind leg and in the intestines to intravenous infusion of increasing amounts of adrenaline.

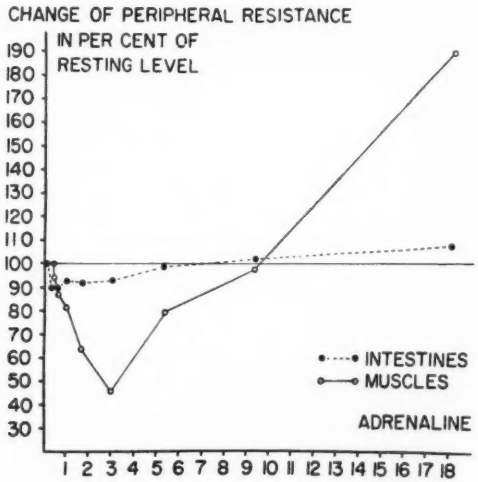

Fig. 8. Changes in peripheral resistance in the experiment shown in Fig. 7.

in the muscle blood flow during noradrenaline infusion are seen in Fig. 2 (the same cat as in Fig. 1).

In the two other vascular areas investigated (skin and intestines) adrenaline showed no vasodilator action. In the skin vasoconstriction appeared after infusion of about 5—15 μg/kg/min (a typical experiment is illustrated in Fig. 5 and 6). Surprisingly, the doses of adrenaline used in this experiment seemed unable to elicit any vasoconstrictor effects in the intestines as judged from the calculations of the peripheral resistance (Fig. 7 and 8). In two similar experiments the peripheral resistance in the intestines increased during adrenaline infusion but only slightly. The peripheral resistance in the intestines did not exceed 110—120 per cent of the resting level, even when large amounts of adrenaline were infused. The problem is further dealt with hereafter.

Discussion

During the course of our investigation of the sympathetic vasodilator outflow we observed now and then that intracerebral stimulation produced vasodilator responses that were partly resistant to atropine. Such a resistance to atropine was inconsistent with a cholinergic nature of the postganglionic vasodilator fibres, and a reasonable explanation was sought. One rather obvious possibility is that vasodilator activity is accompanied by an activation of the adrenal medulla. Investigating this possibility GRANT *et al.* (1958) showed that sympathetic vasodilator activity brought about by hypothalamic stimulation regularly produced a simultaneous discharge of catechols from the adrenals. Adrenaline dominated this discharge, but as long as stimuli of only moderate intensity were applied, the adrenaline output — judging from the present results — was too low to exert any noticeable vasodilator action. The mean value for the adrenaline output from one adrenal during hypothalamic stimulation was 0.32 μg/kg/min. More intense stimulation, however, caused a release of enough adrenaline to produce vasodilation. In other words, the occurrence of vasodilator responses resistant to atropine might well be explained as secondary to the catechol discharge from the adrenals.

Also in medullary stimulation of the sympathetic vasodilator pathway the accompanying adrenaline discharge from the adrenals was too low, as a rule, to produce any vasodilation in the muscles (LINDGREN, ROSÉN and UVNÄS 1959). In this case the adrenaline secretion rose from 0.10 to 0.22 μg/kg/min (mean values from one adrenal gland); only in one experiment where very intense stimulation was applied did it amount to more than 0.50 μg/kg/min.

Noradrenaline did not show any direct vasodilator action. The initial increase in muscle blood flow during noradrenaline infusion, also described by HATCHER and JENNINGS (1957), is probably not due to a vasodilator action but secondary to the initial sharp blood pressure rise. No such response is

seen on intraarterial injection of noradrenaline (LINDGREN, unpublished observations). The vasoconstrictor threshold dose of noradrenaline — about 1 μg/kg/min — indicates that the noradrenaline discharge observed on hypothalamic activation of the sympathetic vasodilator nerves with intense stimulation might add its vasoconstrictor action to the vasomotor reaction pattern.

Quantitative data in the literature on the vasodilator action of intravenous adrenaline infusion are scarce. Observations by HATCHER and JENNINGS (1957) on dogs under sodium pentobarbital and by LUNDHOLM (1957) on cats under Dial anaesthesia agree fairly well with ours. BARCROFT and SWAN (1953) report that in non-anaesthetized human beings adrenaline produces an increase in the forearm blood flow in a dose of 5—10 μg/min.

The fairly high threshold values for the constrictor action of adrenaline on the intestinal vessels are surprising to us. In contrast to what occurs in a cutaneous area, no increase in the peripheral resistance was observed even with doses that gave marked pressor responses. One explanation that has been suggested is that the relaxation of the intestinal muscles would counteract the direct vascular effect of adrenaline. There is no doubt, however, that adrenaline exerts a vasoconstrictor action even in the intestines, otherwise the depressor reflexes that must have been elicited by the blood pressure rises would have caused an intestinal vasodilatation and a reduction of the peripheral resistance in this area.

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Dopamine and Chromaffin Cells

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Abstract

Bertler, Å., B. Falck, N.-Å. Hillarp, E. Rosengren and A. Torp. Dopamine and chromaffin cells. *Acta physiol. scand.* 1959. *47*. 251—258. — The dopamine content of tissues in ruminants (cow, sheep, goat) varies with the numbers of a special type of chromaffin cells and there is a close correlation between the distribution of dopamine and these cells within certain organs. Dopamine — but no other catechol amines or 5-hydroxytryptamine — occurs in concentrations sufficient to produce the observed chromaffin reaction. The findings support the view that dopamine is stored in the special chromaffin cells.

From the findings on the distribution of noradrenaline and dopamine in the bovine lung Euler and Lishajko (1957, 1958) concluded that, in contrast to what is generally believed, dopamine, at least in this tissue, cannot merely be an intermediate metabolite in the synthesis of the adrenergic transmitter.

Using new methods for determination of catechol amines (Carlsson and Waldeck 1958, Bertler, Carlsson and Rosengren 1958) a systematic search for their occurrence has been made in the present and other studies (Bertler and Rosengren 1959 a). The results not only strongly support the view of Euler and Lishajko but also yield evidence that the dopamine found in high concentrations in various tissues of ruminants are stored in special chromaffin cells (cf. Falck, Hillarp and Torp 1959 a).

Methods

Determination of catechol amines and 5-hydroxytryptamine. The determinations were carried out in the way described in previous papers (CARLSSON and WALDECK 1958, BERTLER, CARLSSON and ROSENGREN 1958, BERTLER and ROSENGREN 1959 b).

Histological methods. The tissues were examined with the methods described in previous studies (FALCK, HILLARP and TORP 1959 a, b). Tissue pieces were usually taken adjacent to those used for amine determination.

In an attempt to obtain more exact information on the correlation between the occurrence of dopamine and the special chromaffin cells, cell counts and amine determinations were made using cow liver capsule, which contains abundant chromaffin cells easy to count thanks to their localization as a thin layer in the inner part of the capsule. On the surface of a large liver lobe a square 10 × 10 cm was marked out. Six rectangular pieces of the capsule (length = 10 mm) with some underlying parenchyma were cut out from this area and used for histological examination. The remaining part of the capsule was dissected from the parenchyma and the total amount of dopamine and noradrenaline determined. Chromaffin cells were counted in 20 transverse sections (10 μ thick) from each piece. The sections were taken at different depths of the preparations. The number of cells per cm² was calculated from the number of cells per section. Though only cells with the major part of the nucleus within the section (the cells are 6 to 10 μ in diameter) were counted, the observed values may be somewhat high. Since the pieces were embedded in paraffin to secure a more reliable section thickness some of the cells, however, probably escaped counting owing to the impairment of the chromaffin reaction on paraffin embedding (cf. FALCK and HILLARP 1959). These errors may have neutralized each other, at least to some extent. The shrinkage of the capsule was about 20 per cent, as judged from the length of the sections. Since the pieces were cut out at random as regards the orientation of their long sides on the surface of the liver, it is obvious that each 10 μ section corresponded to a section with a thickness of 12 μ in the fresh tissue. The observed values are thus about 20 per cent too high.

Results

Dopamine and special chromaffin cells in non-ruminants

At most small amounts of dopamine have been found in peripheral tissues in animal species other than ruminants (BERTLER and ROSENGREN 1959 a). Several organs and tissues, especially lung and liver, from non-ruminants of the same kinds as those used in that study were examined histologically, but no chromaffin cells of the special type present in ruminants were found. The cat and rabbit duodenum may contain some dopamine (0.1 μg/g) but no chromaffin cells of the ruminant type were detected. The horse lung showed neither dopamine nor the special chromaffin cells.

Dopamine and special chromaffin cells in ruminants

Some of the tissues in the ruminants examined (cow, goat, sheep) contain high concentrations of dopamine and likewise large numbers of the special chromaffin cells. The cells are, however, not evenly distributed within these tissues, but are concentrated to certain parts in an unexpected way which facilitated the attempts to correlate dopamine content and cell numbers.

Table I. Content and distribution of dopamine (DA) and noradrenaline (NA) in the lung. The figures are µg/g wet weight

	Whole lung		Distribution within the same lung					
			Parenchyma		Visc. pleura		Interlob. conn. tissue	
	DA	NA	DA	NA	DA	NA	DA	NA
Cow.....	3,5	—	2,4	0,0	6,4	0,1	11,5	0,2
	4,8	0,0	3,0	—	14,5	—	14,5	—
	9,5	0,1	4,7	0,2	9,8	0,2	—	—
	15,0	0,1	3,7	0,3	6,7	0,3	—	—
Goat	—	—	2,7	0,1	9,0	0,0	—	—
	—	—	5,3	0,2	10,0	0,1	—	—
Sheep	3,6	0,0	1,7	0,0	3,2	0,0	—	—
	6,9	0,1	—	—	—	—	—	—
	9,8	—	—	—	—	—	—	—

In the *lung* the chromaffin cells are scarce in the intralobular tissue and in the walls of blood vessels, fairly abundant in the thinner connective tissue septa and are present in large numbers in some parts of the thicker connective tissue septa, especially in the hilus. They are, however, most regularly concentrated to the visceral pleura, especially in the cow. Dopamine is distributed in the same peculiar way, the concentrations being from two to five times higher in the pleura and in some connective tissue than in the parenchyma (Table I).

An even more striking correspondence was found in the cow *liver*, in which practically all chromaffin cells are located as a thin layer in the inner part of the capsule, only isolated cells being present in the connective tissue within the liver. Dopamine is also highly concentrated in the capsule (Table II). In the sheep the distribution is similar but the chromaffin cells are generally fewer and scattered in the capsule. Only three goats were examined histologically but all showed many chromaffin cells in the interstitial connective tissue of the liver and even some cells within the lobules. This agreed well with the dopamine determinations showing higher concentrations in the parenchyma than observed in the cow and sheep (Table II). Furthermore, in one of the goats (the first in the table) rather few cells and low dopamine content was found in the capsule.

A third large storage site for dopamine was found in the *intestine*, especially the duodenum (Table III). The bulk of the amine is stored in the mucosa or submucosa, as judged from determinations on the mucosa membrane and muscularis separately (a single observation on cow duodenum not shown in the table). Abundant chromaffin cells, differing from the enterochromaffin cells (see FALCK *et al.* 1959 b) and of the same type as those present in the lung

Table II. Content and distribution of dopamine (DA) and noradrenaline (NA) in the liver. The figures are μg/g wet weight

	Whole Liver		Distribution within the same liver			
	DA	NA	Capsule		Parenchyma	
			DA	NA	DA	NA
Cow.....	2,5	0,1	17,0	0,2	1,75	0,3
	2,5	0,0	7,9	0,1	0,55	0,1
			7,8	0,5		
			6,9	0,4		
Goat			1,6	0,2	1,6	0,1
			7,3	0,5	1,6	0,1
Sheep	0,3	0,2	1,4	0,4	0,1	0,2

Table III. Content (μg/g wet weight) of dopamine (DA) and noradrenaline (NA) in the intestines

	Duodenum		Ileum		Colon	
	DA	NA	DA	NA	DA	NA
Goat	6,3	0,2	4,0	0,2	2,5	0,2
	2,1	0,4	2,0	0,2	1,9	0,2
Cow.....	4,4	0,1				
Sheep	4,2					

and liver but showing a stronger chromaffin reaction, were found to occur in the intestines. Their numbers are highest in the cranial part but many are present also in the colon (intestines from cow have not been examined). In the goat they are located in the lamina propria around the bottom of the crypts, but in the sheep mainly in the submucosal connective tissue.

Confirming the observations of SCHÜMANN (1956) and EULER and LISHAJKO (1957) fairly high dopamine concentrations were found in splenic *nerves* from the cow (about 2.5 μg/g). These nerves also contain the special chromaffin cells but markedly fewer than the cow pleura. They lie scattered throughout the nerves and are often compressed between the nerve fibres. Unexpectedly the sciatic nerve was found to contain dopamine in concentrations that, though low, were several times higher than those of noradrenaline (Table IV). Here, too, chromaffin cells are present but they are scarce and mainly located in the perineural sheath.

Other tissues. Several tissues showed low dopamine concentrations (Table IV) and only few, scattered chromaffin cells. The only organ in which no cells

Table IV. Content (μg/g wet weight) of dopamine (DA) and noradrenaline (NA) in various organs and tissues

	Cow		Sheep		Goat	
	DA	NA	DA	NA	DA	NA
Heart (whole)	1,5	1,0	0,3	1,0	0,7	1,4
	—	—	—	—	0,5	1,4
Pericardium (visceral)	0,7	0,1	—	—	—	—
Spleen (whole)	0,8	1,0	0,9	1,9	1,0	4,6
	—	—	—	—	1,0	6,8
Spleen (capsule)	0,4	0,7	—	—	—	—
	0,6	0,2	—	—	—	—
Abdominal muscle ..	—	—	0,2	0,0	0,4	0,0
	—	—	0,15	0,0	1,0	0,1
Third stomach	—	—	—	—	0,2	0,1
Sciatic nerve	—	—	—	—	0,5	0,1
	—	—	—	—	0,4	0,1

were detected was the third *stomach* of the goat (the other two were not examined). This organ contained very little dopamine. In the cow *heart* few chromaffin cells were seen in the endocardium, pericardium and in the interstitial tissue of the musculature. This hardly seems to agree with the amine content. However, since only small pieces were examined microscopically, there may have been larger numbers of cells in other parts of the hearts. Another discrepancy was the fewness of cells in the *spleen* though the amine content was by no means low. Here, however, the closely packed spleen cells and red blood cells make it almost impossible to identify the chromaffin cells.

Several other tissues than those recorded in the tables have been examined histologically. No accumulations of chromaffin cells were detected — with one exception — but isolated cells were scattered almost everywhere throughout the *interstitial connective tissue*. The exception was the inner abdominal muscle layer where abundant numbers of fairly strongly chromaffin cells occurred in one of the several sheep examined.

It should perhaps be mentioned that in four young sheep the chromaffin cells demonstrable in lung, liver and intestine were few in number, probably owing to a weakened chromaffinity. The dopamine content in the tissues of these animals was unusually low.

Dopamine content and numbers of chromaffin cells in cow liver capsule.

With the use of the cow liver capsule it is possible to estimate, at least approximately, the dopamine concentration that should exist in the chromaffin cells if the amine is stored in these cells. This is an essential point since the values

Table V. Dopamine content and numbers of chromaffin cells in cow liver capsule

Experiment	Numbers of cells per section	Numbers of cells per cm²	Dopamine content	
			µg/cm²	µg/cell
I	32—90 M=52	52 000	0,20	$0{,}38\times10^{-5}$
II	25—63 M=39	39 000	0,21	$0{,}54\times10^{-5}$
III	43—82 M=60	60 000	0,28	$0{,}47\times10^{-5}$

obtained may verify or exclude the *possibility* that the chromaffinity of the cells is due to the presence of dopamine.

Dopamine is readily oxidized with bichromate to insoluble dark pigments (HILLARP and HÖKFELT 1953) and — like adrenaline and noradrenaline — may thus give a chromaffin reaction in cells if present in sufficiently high concentration. The chromaffin cells in the adrenal medulla have an amine content of about 1 to 1.5 per cent but — as judged from observations in our laboratories on medullary cells with decreased amine content — a weak chromaffin reaction may probably be obtained even if the concentration is so low as 0.3 per cent. The special chromaffin cell in the cow liver capsule may contain 0.4 to 0.5 $\times 10^{-5}$ µg of dopamine (Table V). The cells have a diameter of about 6 to 10 µ in histological preparations but due to shrinkage their true size is probably about 10 µ. The concentration of dopamine may thus be 0.7 to 0.9 per cent. Since the calculated values for the cell numbers are probably too high (see Methods), the concentration might be higher, but, on the other hand, the assumed size of the cells might be somewhat too small. At any rate the calculated figures for the dopamine concentration, even if thought to be as much as two times too high, clearly show dopamine in cow liver capsule, if stored in the special cells, to be present in concentrations sufficient for a chromaffin reaction.

Discussion

The results indicate that the dopamine found in peripheral organs of ruminants is stored in a special type of chromaffin cells. There is no reason to believe that the chromaffinity of the cells is due to adrenaline, noradrenaline or 5-hydroxytryptamine (5-HT) which are other substances known to give chromaffin reaction.

Adrenaline and noradrenaline can be excluded since the concentrations of these amines in the examined tissues are too low to account for the chromaffin reaction. In the visceral pleura, liver capsule and duodenum, especially in the cow, the content of noradrenaline is below that of dopamine by a factor of ten

to hundred (Tables I to III). Adrenaline in most tissues is present in concentrations less than 10 per cent of those of noradrenaline. This is in accordance with the observations of EULER and coworkers (cf. EULER 1956).

Nor does the chromaffinity seem to be due to 5-HT. Many tissues of non-ruminants have high concentrations of 5-HT but do not contain the special chromaffin cells. On the other hand certain tissues, *e. g.* the lung and liver capsule of the cow, which are rich in chromaffin cells, contain but little 5-HT.

DOPA (3,4-dihydroxyphenylalanine) is another substance which might give the same histochemical reactions as dopamine, but it occurs in tissues in very low concentration, if present at all. The spectrophotofluorometric examinations of extracts from cow lung do not reveal any other catechol amine which can account for the chromaffin reaction.

The histochemical reactions also are in agreement with the assumption that the chromaffin reaction is due to dopamine and seem to exclude that it is due to adrenaline and 5-HT (FALCK *et al.* 1959 b).

The chromaffin cells recently discovered by ADAMS-RAY and NORDENSTAM (1956), NORDENSTAM and ADAMS-RAY (1957) may be present in for example the cow liver capsule, though only scarcely seen by us, but these cells occur also in non-ruminants and seem to be distributed like the vasomotors (ADAMS-RAY: personal communication). Mast cells are abundant in the pleura and liver capsule in the cow and since they are known to store histamine (cf. WEST 1956) and in some animals even 5-HT (BENDITT 1958) dopamine might possibly also be stored in these cells. It was found, however, that the distribution of mast cells within the cow lung did not follow that of dopamine. Thus, there were about as many cells in the intralobular tissue as in the visceral pleura (staining according to HOLMGREN).

Summary

1. The dopamine content of tissues in ruminants (cow, sheep, goat) varies with the numbers of a special type of chromaffin cells and there is a close correlation between the distribution of dopamine and these cells within certain organs. The tissues of non-ruminant animals which contain at most small amounts of dopamine lack chromaffin cells of this type.

2. Dopamine and chromaffin cells are widely distributed but are localized mainly in the lung, liver and small intestines. The liver capsule and visceral pleura, especially in the cow, show the highest concentrations.

3. Determinations of the dopamine content and the numbers of chromaffin cells in the cow liver capsule showed that dopamine occurs in concentrations sufficient to produce the observed chromaffin reaction. The chromaffinity of the cells cannot be due to the presence of adrenaline, noradrenaline, dopa or 5-hydroxytryptamine, their concentrations being much too low in several of the tissues examined.

4. The findings in the present study together with those in a previous study on the histochemistry of the special chromaffin cells strongly support the view that dopamine is stored in these cells.

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Acta physiol. scand. 1959. 47. 259—261

From the Department of Histology, University of Lund, Lund, Sweden

A Simple Device for Long-Lasting, Continuous Injections into Animals

By

B. Falck, N.-Å. Hillarp and G. Thieme

Received 6 April 1959

Abstract

Falck, B., N.-Å. Hillarp and G. Thieme. A simple device for long-lasting, continuous injections into animals. *Acta physiol. scand.* 1959. *47*. 259—261. — An injection syringe to be carried by the animal has been devised. The syringe allows continuous injection of given volumes of fluid in given periods of time (from half a day up to several days). The injection fluid is pressed through a filter delaying the outflow and the rate of the outflow may be varied by varying the viscosity of the fluid.

A slow and continuous absorption of substances may be secured by implantation of the substances in the form of pellets. It is often difficult, however, to find suitable vehicles for many substances, especially for those readily soluble in water. Furthermore, it may be almost impossible to produce pellets that will permit an even absorption of the whole amount of a certain substance during a certain period of time. A special injection syringe to be carried by the animal was therefore devised. The device allows continuous injection of given volumes of fluid in given periods of time (from half a day up to several days).

In principle the device is a syringe with a spring-loaded self-sealing piston which presses the injection fluid through an outlet containing a filter delaying

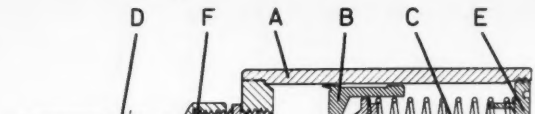

Fig. 1.

the outflow. The rate of the outflow is varied by increasing or decreasing the viscosity of the injection fluid.

The data given below are the measures for a 5 ml syringe suitable for use on rabbits, for example. All metal parts are made of stainless steel. The cylinder (A in Fig. 1) is made of a Perspex tube, the inner surface of which is ground and polished. As material for the piston (B) nylon proved unsuitable because of its tendency to swell: therefore polythene was chosen. The front surface of the piston is provided with a thin flange, which is forced against the wall by the pressure of the fluid to be injected. The flange therefore serves as a self-sealing packing.

The coil-spring (C), made of 1.0 mm steel wire, has a length of 65 mm when unloaded. To prevent asymmetric deformation of the fairly soft polythene piston, the end of the spring is fastened to a metal cone fitting a conical hollow in the centre of the piston.

The filter (D), which delays the outflow, is packed in a steel tube with an inner diameter of 2.6 mm and a length of 30 mm. A polythene packing (F) is used to secure a snug fit between the tube and the front nut. The filter is made of glass wool which is packed very tightly in the tube. The fibres of ordinary glass wool are too thick to provide sufficient resistance. A special glass wool with superfine fibres (thickness of 1 to 2 μ) was found suitable (manufactured by Billesholms Glasulls AB, Billesholm, Sweden). Once used the filter must be kept moist, drying changing its flow resistance.

The rate of outflow may be controlled in several ways. Thus the diameter, length or compactness of the glass fibre filter may be varied. Since a very tightly packed filter (obtained by hammering on a metal stopper) seems to give the most reliable results, the best and simplest method is, however, to increase the viscosity of the injection fluid by adding a suitable liquid or substance (glycerol or dextran, for example). In this way injection of 5 ml of fluid may be spread over more than a week.

The use of a very tightly packed filter is also important for another reason. Such a filter has a powerful delaying effect, so that it is usually unnecessary to use high viscosities. If liquids with high viscosity are used, the increase in temperature of the syringe and its contents owing to heat from the animal's body may result in a considerable increase in flow rate. Corrections for temperature differences must therefore be made when using such liquids.

The syringe is most suitably handled in the following manner. After removing the back screw (E), the filter tube is unscrewed and the piston pushed half-backwards. The injection fluid is then transferred to the syringe and the piston is pushed forwards to level the liquid to the front nut. After replacing the filter tube the coil-spring and back screw are inserted again. Since the spring pressure is about 1.5 to 2 kg, the last step may be facilitated by guiding the back screw with a special key.

The syringe is fastened to the animal by adhesive plaster and sutures for example. The syringe may be conveniently connected to the desired site of injection by means of a thin flexible catheter.

Acta physiol. scand. 1959. 47. 262—270

From the Department of Physiology, University of Göteborg, Göteborg, Sweden

A Hypothalamic Structure with a Marked Inhibitory Effect on Tonic Sympathetic Activity

By

BJÖRN FOLKOW, BÖRJE JOHANSSON and BENGT ÖBERG

Received 13 April 1959

Abstract

FOLKOW, B., B. JOHANSSON and B. ÖBERG. A hypothalamic structure with a marked inhibitory effect on tonic sympathetic activity. *Acta physiol. scand.* 1959. *47*. 262—270. — In cats the brain has been explored with the Horsley-Clarke technique in a search for hypothalamic structures from which distinct inhibitions of sympathetic activity can be elicited. Just behind and below the anterior commissure, about 2 mm from the midline, a restricted area has been localized, from which often drastic inhibitions of sympathetic vasoconstrictor-accelerans tone can be induced. It is suggested that this structure constitutes a hypothalamic relay station for cortical inhibitory pathways to subordinated sympathetic structures, mainly affecting the discharge of the medullary vasomotor centre.

For several decades extensive topic stimulations of the hypothalamic area have been performed and the effect on the cardio-vascular system has been especially studied. On the basis of the effects on the blood pressure and the pulse rate it has generally been stated that 'depressor areas' are concentrated in anterior parts and 'pressor areas' in posterior parts of the hypothalamus (*e. g.* RANSON and MAGOUN 1939, HESS 1948). Conclusions regarding the more detailed cardio-vascular events can, however, only exceptionally be drawn from experiments of this type, as, for example, most dramatic neurogenic redistributions of blood flow with great shifts in cardiac output can occur at very little change of pressure (see for instance ELIASSON *et al.* 1951).

The often marked pressor effects, obtainable especially from posterior hypothalamic parts, are for good reasons considered to be due mainly to increases of sympathetic vasoconstrictor and accelerans fibre tone. The nature of the hypothalamic depressor effects is then often more varied with regard to its background. At least three different types of neurogenic cardiovascular mechanisms can here be engaged, not taking into account such depressor effects that are simply caused by respiratory changes or skeletal muscle activation. Thus the blood pressure fall can be due to excitation of specific vasodilator fibres, to activation of vagal fibres to the heart and/or to a generalized, or regional inhibition of sympathetic vasoconstrictor-accelerans tone. Stimulation of the hypothalamic relay centre for the sympathetic cholinergic vasodilator fibres can, for instance, sometimes induce fairly pronounced depressor effects, though the muscular vasodilatation is in most cases well balanced by constrictions in other areas and by a sympathetic activation of the heart (Eliasson *et al.* 1951). — It has often been observed, that hypothalamic stimulation induces blood pressure falls with marked bradycardia and concomitant activation of the gastrointestinal tract, where the depressor effects are more or less abolished by vagotomy (*e. g.* Beattie and Sheehan 1934, Kabat, Magoun and Ranson 1935, Korteweg, Boeles and Ten Cate 1957). — It has also been postulated that, beside the sympatho-excitatory areas, there should exist centres in the hypothalamus that cause inhibition of sympathetic constrictor fibre tone. Little is, however, at present known about such sympatho-inhibitory centres, even if there are some data to indicate their existence. For instance, in a series of electrophysiological analyses of the hypothalamic influence on sympathetic activity, Pitts, Larrabee and Bronk (1941) observed that a moderate inhibition of the tonic sympathetic activity to the heart could sometimes be obtained at low frequency stimulation of the hypothalamus. They did not, however, identify a characteristic part of the hypothalamus from which this reaction could be more regularly elicited. In their extensive hypothalamic exploration Kabat *et al.* (1935) were only in a few cases able to induce depressor responses that could be ascribed to an inhibition of sympathetic activity.

In the present investigation a restricted hypothalamic area, from which a marked inhibition of the tonic discharge in the sympathetic vasoconstrictor-accelerans fibres can be induced, has been localized and the elicited cardiovascular reactions analyzed.

Methods

The experiments were performed on cats, lightly anaesthetized, generally with 30—40 mg chloralose per kg body weight. The calvarium was exposed, and a part of the skull, 10 mm in square, just behind the coronal suture was removed with a drill. By means of a Horsley-Clarke instrument concentric, bipolar electrodes, insulated except at their tips, were bilaterally introduced for stimulation of corresponding areas

of the hypothalamus on both sides of the midline. The stimuli were delivered by a Grass stimulator, at a voltage varying between one and three, a pulse frequency between ten and hundred per second and a pulse duration of 1—2 msec. — The animals were heparinized and the blood pressure was recorded from the left femoral artery by means of a mercury manometer. Blood flow in different vascular areas was measured by a closed optical drop recorder-ordinate writer unit, connected to the draining vein. In this way the cutaneus, muscular or the intestinal blood flow was measured from respectively the right big saphenal vein at the level of the ankle, from the branch of the right femoral vein that drains the muscles in the right calf and lastly from the superior mesenteric vein in a preparation where only a segment of the small intestine was left *in situ*. The blood was returned to the animal by way of a cannula in the left femoral vein. — The heart frequency was estimated either by a pulse recorder operating an ordinate writer or simply by counting the pulse. — To avoid disturbing influences from changes in activity of the skeletal muscles and respiratory movements, decamethonium, 0.5 mg per kg body weight, was given intravenously in the majority of the experiments. Constant artificial respiration, previously so adjusted that it barely suppressed spontaneous respiratory movements, was then given. — In order to eliminate the effects of the cholinergic vasodilator fibres and the vagal fibres to the heart the vagal nerves were cut and atropine, 0.3—1 mg per kg body weight, was given intravenously. The completeness of the atropine blockade was tested by injection of a large dose of acetylcholine or by stimulation of the peripheral end of the vagal nerves. — In such a preparation the central nervous influence on the cardiovascular system is restricted to changes in the discharge of the sympathetic vasoconstrictor and accelerans fibres. To create a high initial sympathetic activity and also in order to eliminate the buffering effect of the baro- and chemoreceptor reflexes one, or sometimes both of the carotid arteries were occluded before the hypothalamic stimulation. To prove that the depressor reactions really were caused by a change in sympathetic vasoconstrictor tone, the hypothalamic stimulations were repeated also after the different vascular beds, in which the blood flow was recorded, had been sympathectomized, or alternatively their vasoconstrictor fibres blocked by close intra-arterial injections of dihydroergotamine, generally about 0.2—0.4 mg, slowly injected into the artery of the studied tissue.

At the end of the experiments the points of hypothalamic stimulation were marked out either by small electrolytic lesions or by painting the electrode tips with india ink, after which they were again inserted in the stimulated area. The brains were then fixed in a 10 % solution of formaline, and the points of stimulation were determined under low magnification.

Results

As seen from Fig. 1 weak electrical stimulation of a strictly localized area in the anterior hypothalamus markedly inhibits the discharge of the sympathetic fibres to the cardiovascular system, reflected in the figure as a fall in blood pressure of 105 mm Hg and a 20 per cent decrease in pulse rate. In some animals the blood pressure fell rapidly at the onset of stimulation, in others the fall was quite sluggish, though once it had reached its maximum its magnitude could be just the same. A characteristic feature was the slow restitution of the blood pressure after the interruption of the stimulation, often lasting

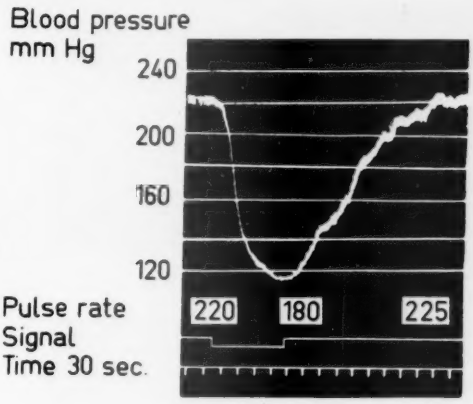

Fig. 1. Cat, 3.2 kg. Chloralose. The effect of stimulation in the hypothalamic sympatho-inhibitory area on arterial blood pressure. The shift in heart rate is also given. Stimulation characteristics 3 volts, 1 msec, 60 impulses per second.

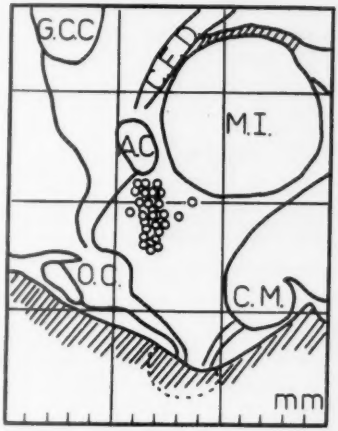

Fig. 2. Medial sagittal section of the diencephalon, showing the location of the hypothalamic area in which stimulation causes inhibition of sympathetic tone. AC: anterior commissure; CFD: Columna fornicis descendens; CM: mammillary bodies; GCC: Genu of corpus callosum; MI: Massa intermedia; OC: Optic chiasm.

3—5 min or more, which indicates a prolonged inhibition of vasoconstrictor fibre tone.

In Fig. 2 the stimulation points in the majority of the experiments are mapped out, indicating that the inhibitory structure is situated just behind and below the anterior commissure, about 2 mm laterally to the midline. The scatter of the stimulation points in different experiments, shown in Fig. 2, is probably not a matter of a big extension of the depressor area but due to errors in the estimation of the actual electrode position and to minor differences in the localization of the area from one animal to another. The region seems in fact to be very restricted in its extension, as in any given experiment

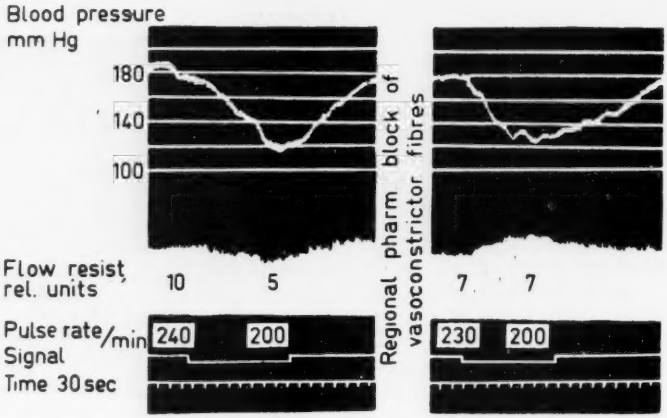

Fig. 3. Cat, 3.5 kg. Chloralose. Records of arterial blood pressure and blood flow in the calf muscles with the effects of hypothalamic stimulation before and after pharmacological block of the sympathetic vasoconstrictor fibres. In this, as in the subsequent figures, the absolute shifts in heart rate and the relative shifts in regional resistance to flow are also given. Stimulation characteristics 2.5 volts, 1 msec, 70 impulses per second.

the depressor responses were either markedly decreased, abolished or changed to pressor effects by moving the electrode only 1 mm in either direction. This is also supported by the fact that on even fairly moderate increases of voltage, which imply a bigger spread of the stimulation current, the depressor response was either decreased or, at a still higher voltage, even reversed to a pressor response, obviously due to an increasing activation of adjacent excitatory pathways.

The depressor effects and the decreases in pulse rate, obtained from this area, were never reversed by, for example, shifts in pulse duration or stimulation frequency, though of course the magnitude of the blood pressure fall was dependent on the frequency. Rather marked effects were often obtained already at 10—20 impulses per sec and maximal depressor responses were generally reached at 50—60 impulses per sec. Still higher frequencies had mainly the consequence that the depressor response soon decreased in spite of continued stimulation, presumably due to transmission failure somewhere along the inhibitory pathways, as the response reappeared on shift to a lower frequency.

When the inhibitory area had been fairly well localized, attempts were made to analyse in more detail the cardiovascular events. A characteristic feature in the stimulatory response was the decrease in heart rate, which occurred as rapidly as the fall in blood pressure and often amounted to about 20—25 per cent of the initial heart rate. It was too prompt in onset to be due to an inhibition of a prevailing adrenaline secretion to the blood stream. As the vagal

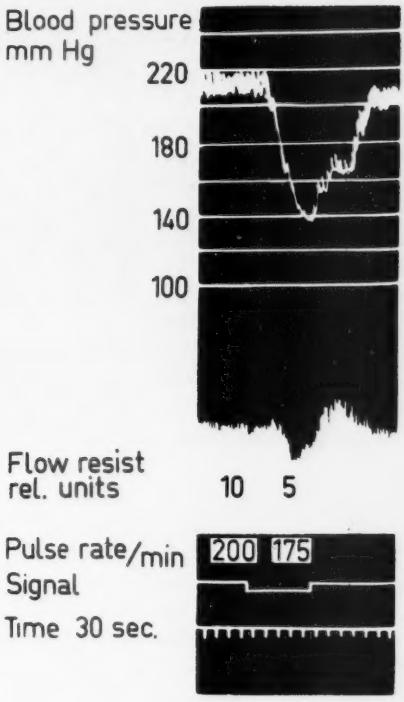

Fig. 4. Cat 3 kg. Chloralose. Records of arterial blood pressure and blood flow in the cutaneus vessels of the hind paw with the effects of hypothalamic stimulation. Stimulation characteristics 3 volts, 1 msec, 60 impulses per second.

nerves were cut these decreases in heart rate indicate an inhibition of accelerans fibre tone. — With regard to blood circulation shifts in different areas, the effect of the hypothalamic stimulation on the regional vascular resistance was estimated from the pressure-flow values (see *e. g.* GREEN *et al.* 1944). Fig. 3, 4 and 5 are representative for those experiments in which the blood flow in respectively the skeletal muscles, the skin of the paw and the small intestine was recorded. As is seen from these figures the regional blood flow could increase somewhat in these experiments in spite of a considerable fall in pressure, indicating a marked fall in the resistance to flow within the tissue, often 50 per cent or more. In other cases there was no change in blood flow, which still means a considerable widening of the vessels as at the same time the blood pressure fell markedly. — After regional sympathectomy or close arterial injections of dihydroergotamine the blood flow decreased regularly in proportion to the blood pressure fall, induced by hypothalamic stimulation, indicating that the vascular widening was now abolished in the studied vascular area (see Fig. 3, 4 and 5). — If dihydroergotamine 0.5—1 mg/kg was administered i. v., so that it blocked the effect of the constrictor fibres in all

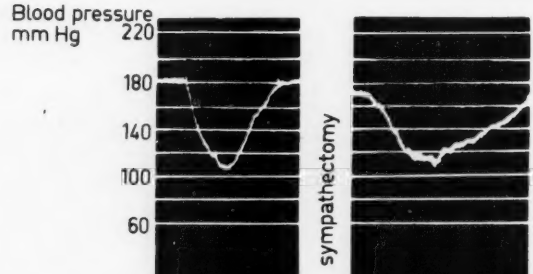

Fig. 5. Cat 3.6 kg. Chloralose. Records of arterial blood pressure and blood flow through an intestinal loop with the effects of hypothalamic stimulation before and after regional sympathectomy. Stimulation characteristics 2 volts, 1 msec, 70 impulses per second.

vascular regions, the depressor effects on hypothalamic stimulation were abolished. On the other hand, cholinergic blocking drugs, like atropine, if not given beforehand, did not affect the depressor responses elicited from the studied hypothalamic area.

Taken together, the results indicate that from the studied hypothalamic area a dramatic and fairly generalized inhibition of the tonic activity of sympathetic adrenergic fibres is elicited without any concomitant engagement of vasodilator fibres. The inhibitory area is, however, situated quite near the hypothalamic relay centre of the cholinergic sympathetic vasodilator fibres (ELIASSON *et al.* 1951), obvious from the fact that if atropine is not given beforehand and the voltage is somewhat increased, a downward shift in electrode position of only 1—2 mm can change the generalized inhibition of vasoconstrictor fibre tone to a drastic increase of only the muscle blood flow with just a little shift in blood pressure. Atropine abolished this muscle vasodilatation but left the inhibitory response, obtained 1—2 mm more dorsally in the hypothalamus, unchanged.

Discussion

The depressor effects which earlier investigators have observed on electrical stimulations of anterior parts of the hypothalamus have usually been considered to be the result of parasympathetic activation. The existence of

hypothalamic sympatico-inhibitory 'centres' has been suggested, but experimental evidence supporting such a view has so far not been clearcut (for lit. see FOLKOW 1956).

In the present series of experiments the existence of a restricted hypothalamic structure, from which marked inhibitions of sympathetic tone can be elicited, has been demonstrated and its influence on the cardiovascular system has been analyzed. The inhibitory area is situated just behind and below the anterior commissure and takes up an obviously very small section of the hypothalamus. In animals, so prepared that other neurogenic cardiovascular influences were excluded, the inhibitory effect on sympathetic activity manifested itself as an often marked blood pressure fall, a decrease in heart rate and an obviously generalized decrease of vasoconstrictor fibre tone as judged from the changes in peripheral resistance to blood flow in different vascular beds. The response elicited was throughout an inhibitory one, independent of stimulation frequency, pulse duration and voltage, as long as the spread of current was not extensive enough to excite more significantly also surrounding sympatho-excitatory areas of the hypothalamus.

In some animals it proved difficult or even impossible to induce more clearcut depressor effects, but it should be remembered that it is often quite difficult to obtain an exact electrode position, which is necessary as the inhibitory area appears to be very restricted in its extension. Accidental concomitant stimulation of adjacent excitatory structures can, of course, easily mask the effect of less complete excitations of the inhibitory area. It was further observed that the animal's general condition was of great importance. Thus, more intense depressor responses were obtained only in superficially anesthetized animals, and if increased amounts of anaesthetics were given, the responses were often diminished or even abolished. It is therefore probable that still more pronounced inhibitory effects can be induced by topical stimulation in conscious animals. The restricted dimensions of the sympatho-inhibitory area and the importance of having optimal experimental conditions may explain why in earlier explorations of the hypothalamus it seems to have escaped detection.

From the present experiments it can hardly be decided whether the studied inhibitory area is a synaptic relay station or simply a section of an efferent fibre tract, which in this part of the hypothalamus happens to form a spatially well-defined bundle. If the latter alternative were the case, one should, however, expect that it should be possible to trace well-defined inhibitory responses at least for some distance along the course of the fibres. This was hardly the case, and this circumstance may favour the first mentioned alternative, *i. e.* that it should constitute a hypothalamic relay-station for cortical sympatico-inhibitory pathways where the incoming and outgoing fibres are so scattered, or form such delicate bundles, that they are difficult to activate selectively by the utilized technique.

Little is known about the further course of the inhibitory pathway down

through the brainstem. The fibres might here run mainly in the medial parts of the mesencephalon as has been suggested by THOMPSON and BACH (1950). Further experiments with partial sections of the brainstem may clarify this question. It would also be of interest to know whether the inhibitory effect is exerted upon the neurons of the medullary vasomotor centre, or if possibly the inhibitory fibres make contact with the preganglionic sympathetic neurons in the spinal medulla.

So far it is not known with any certainty under which circumstances the hypothalamic sympatho-inhibitory area is activated. It may, as suggested above, form the diencephalic relay station for a pathway system by which cortical structures exert an inhibitory influence on the activity of hypothalamic and medullary sympathetic structures. The sham rage phenomenon in decorticate animals indicates that the cerebral cortex exerts an important inhibitory control on lower sympathetic centres. It is known, for instance, that damage to the orbital cortex alone can result in a release of sympathetic activity (KENNARD 1945). The autonomic fibres from this cortical area appear to run to the anterior hypothalamus (WALL and DAVIS 1951). Possibly it is also *via* this hypothalamic structure that some types of psychogenic stimuli affect the cardiovascular system, *e. g.* in emotional fainting.

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Acta physiol. scand. 1959. 47. 271—279

From the Department of Histology, University of Lund, Lund, Sweden

Further Observations on the State of the Catechol Amines Stored in the Adrenal Medullary Granules

By

NILS-ÅKE HILLARP

Received 16 April 1959

Abstract

HILLARP, N.-Å. Further observations on the state of the catechol amines stored in the adrenal medullary granules. *Acta physiol. scand.* 1959. *47.* 271—279, — On incubation of amine granules from cow adrenal medulla in media containing labelled adrenaline no significant exchange between this adrenaline and the amines stored in the granules was found. This and other findings indicate that the stored amines are bound in a non-diffusible state in combination with adenosinephosphates and probably with intragranular proteins. The supposed complex binding the amines seems to be a very labile structure, which is destroyed on disruption of the granule membrane.

The catechol amines in the adrenal medullary granules seem to be stored in an osmotically inactive form (CARLSSON and HILLARP 1958) and are thus probably bound in some way in the granules.

The present study was performed to get further information concerning the state of the stored amines. The experiments indicate that the amines are bound in a non-diffusible form within the granules.

Methods

Isolation of the medullary granules: The catechol amine granules of the cow adrenal medulla were isolated in 0.3 M sucrose by means of centrifugation in the way described in a previous paper (HILLARP 1958 c). The granules were washed twice and then sus-

pended in a small volume of 0.3 M sucrose. The procedure gives a fraction which is but slightly contaminated by mitochondria and microsomes.

Determination of wet and dry weight: 2.0 ml of a granule suspension (about 300 mg of granules) were mixed with 5.0 ml of 0.17 M KCl. The electrolyte was added to secure a compact and firm sediment on centrifugation (38,000 × *g* for 60 min). The main part of the sediment was weighed and dried to constant weight *in vacuo* over P_2O_5. The residue was extracted with 5 % trichloroacetic acid and the proteins and catechol amines were determined.

Dialysis and ultrafiltration: Granules sedimented from sucrose suspensions were rapidly lysed in water at 0° and the insoluble material immediately spun down to prevent the ATPase present in the granule stroma from dephosphorylating the ATP released (cf. HILLARP 1958 a). The supernatant, containing the amines (about 3 mg/ml) and the other soluble content of the granules (adenosinephosphates, proteins and some lipids), was immediately used for dialysis and ultrafiltration. The dialysis was performed at + 20° against an equal volume of water through a cellophane membrane separating two shallow circular compartments designed in such a way as to prevent foaming of the water lysate on rapid shaking. At intervals small aliquots were taken for amine determinations. The ultrafiltration was performed by means of suction through a collodion membrane (Membranfilter, Göttingen). The filtrate was analyzed for catechol amines.

Paper ionophoresis: Water lysates (containing about 5 mg of amines per ml) were examined by paper ionophoresis (10 V/cm; 1 to 12 hours at 0°) using sodium phosphate buffers of pH 6 to 7.5 and ionic strength 0.1. The proteins were developed with bromphenol blue and the catechol amines by oxidation with potassium ferricyanide (JAMES 1948).

Studies on the exchange between the intragranular amines and labelled adrenaline: To 2.0 ml of a granule suspension in 0.3 M sucrose was added 1.0 ml of a solution (0.3 M sucrose) containing tritium labelled adrenaline in varying concentrations. In experiment I (Table II) the solution was brought to pH 7.5 with sodium phosphate, in the two other experiments to pH 6.5 with sodium acetate. The granule suspension was kept at 0° for 24 hours and then centrifuged (38,000 × *g* for 60 min). The supernatant was carefully sucked off, any fluid adhering to the walls was wiped off, and the sediment was weighed. The sediment was resuspended in 7.0 ml of a solution with the same composition as the original suspension medium but without adrenaline. After one hour at 0° the granules were again sedimented and the supernatant removed. The granules were washed twice again in this way. To prevent extraction of any labelled adrenaline adsorbed to the granules, the intragranular amines were released by lysis in 0.015 M KCl. After centrifugation one aliquot of the final supernatant and one from the preceding supernatants were treated with perchloric acid (final concentration 0.4 N). The proteins were spun down, and the extracts were neutralized to pH about 6 with potassium hydroxide. The potassium perchlorate was removed in the cold and the catechol amines in part of the extracts taken up on a small cation exchange column (Dowex 50, 150 to 300 mesh, pH 6). The amines were eluted with N HCl, the eluates evaporated *in vacuo* and the amines dissolved in water. Aliquots were taken to determinations of catechol amines and radioactivity.

Preparation of tritium labelled adrenaline: Adrenaline was labelled with tritium in an apparatus slightly differing from that described by BERGSTRÖM and LINDSTEDT (1957). The labelled adrenaline (10 mg at a time) was taken up on a cation exchange column (Dowex 50, 200 to 300 mesh, 150 × 10 mm, H^+), which was then thoroughly washed with water and one liter of 2 N acetic acid. Elution was performed with N HCl in fractions of 10 ml. Only the top fractions were used. After evaporation of these fractions *in vacuo* the adrenaline was dissolved in water and kept at — 25°.

The labelled product showed an ultraviolet spectrum indistinguishable from that of pure adrenaline, but the adrenaline content determined from the absorption at 280 mμ was about 20 % higher than determined colorimetrically and thus showed the presence of decomposition compounds. The product was examined by high voltage paper electrophoresis (NaAc — HAc buffer of ionic strength 0.1 and pH 4.1 and 6.2, 55 V/cm, 2 hours) and paper chromatography (n-butanol-acetic acid-water 4 : 1 : 5; n-butanol-N HCl 1 : 1; phenol-HCl; see BERTLER, CARLSSON and ROSENGREN 1958). One main compound (about 90 per cent of the value determined colorimetrically) was revealed on oxidation with potassium ferricyanide. It had the same mobility as pure adrenaline and showed, after elution, the same adrenaline content determined both colorimetrically and by ultraviolet absorption. A large portion of the radioactivity was lost, however. The isolated adrenaline showed an activity of about 1,000 cpm/μg (somewhat higher on ionophoresis). This figure was used for the calculations in the exchange experiments.

The labelled adrenaline eluted from the cation exchange column was thus contaminated with high-counting compounds. But since it proved difficult to remove them by ion exchange chromatography and since the results of the exchange experiments (see Results) showed them not to invalidate the interpretations, no further purification seemed necessary.

Analytical methods: Catechol amines were determined according to EULER and HAMBERG (1949) and protein-N by the biuret method of CLELAND and SLATER (1953). The radioactivity measurements were kindly performed by Dr. T. Perklef at the isotope laboratory of AB. Leo, Hälsingborg, and determinations of total lipids by Dr. B. Borgström at the Department of Medical Chemistry, Lund.

Results

Composition of the amine granules

The general chemical composition of the amine granules is shown in Table I. The figures differ somewhat from those obtained previously (HILLARP and NILSON 1954 b) probably owing to the fact that the sediments of amine granules used in the present study were more firmly packed and were contaminated much less by mitochondria and microsomes.

The granule sediment contained extragranular water (about 15 % of the water) and some amounts of sucrose and potassium chloride (cf. CARLSSON and HILLARP 1958). The wet and dry weights observed must therefore be corrected, and in Table I corrected average values are given (bottom row). The contents of adenosinephosphates were not directly determined but calculated from the amine contents according to the results of previous analyses of granules isolated in the same way (HILLARP 1958 b).

The catechol amines, adenosinephosphates, proteins and lipids represent practically the entire content of solids in the granules, since these compounds and water represented 98 % of the granule weight.

Exchange between intragranular catechol amines and labelled adrenaline

In three experiments (Table II) amine granules were suspended in a solution of tritium labelled adrenaline and kept at 0° for 24 hours. After the

Table I. General chemical composition of amine granules isolated from cow adrenal medulla. The average values (bottom row) were corrected for the presence of extragranular water and of sucrose and potassium chloride in the granule sediments

	Wet Weight mg	Water per cent	Catechol amines Per cent		Proteins Per cent		Adenosine-phosphates Per cent		Total lipids Per cent	
			of Wet Weight	of Dry Weight	of Wet Weight	of Dry Weight	of Wet Weight	of Dry Weight	of Wet Weight	of Dry Weight
I	302	69.5	6.0	19.5	9.8	31				
II	278	70.5	5.8	19.5	9.8	33				
III	247	70.0	5.6	18.5	9.8	33				
Average		70.0	5.8	19.0	9.8	32	3.9	13	6	20
» ..... (corrected)		68.5	6.7	20.5	11.5	35	4.5	15	7	22

Table II. Exchange between intragranular amines and labelled adrenaline in the external medium. Amine granules were incubated for 24 hours in 0.3 M sucrose (3.0 ml) containing various concentrations of tritium labelled adrenaline. After the incubation the granules were washed three times

	Total activity: cpm $\times 10^{-3}$				Labelled Adrenaline in suspension Medium µg	Labelled Adrenaline in Washed Granules µg	Catechol Amines in Washed Granules µg
	Suspension Medium	Washings	Intragranular Water (calculated)	Washed Granules			
I ...	1,350	98	105	86	570	86	14,800
II ...	10,800	580	595	130	4,500	130	9,300
III ...	24,700	1,350	1,500	74	10,000	74	7,600

granules had been washed three times the radioactivity of the intragranular amines was determined.

The granule membrane is permeable to adrenaline, so that adrenaline added to the suspension medium penetrates into the intragranular water where it reaches about the same concentration as in the external medium (CARLSSON and HILLARP 1958). This together with the long time used and the short diffusion distances present must be considered to favour an exchange between the stored and added adrenaline. The intragranular amines were, however, found to have only low radioactivity even when the concentration of the labelled adrenaline in the external medium was very high (experiment III in Table II). Further, the figures given for the amounts of labelled adrenaline in the washed granules are maximal values, since much of the activity may

have belonged to decomposition products (see Methods). Thus at most a very small (or very slow) exchange occurred.

The volume of the intragranular water was calculated from the data on the amine content. If the labelled compounds added to the external medium penetrated the granules, the activity in the intragranular water should show the values calculated in Table II (fourth column). The calculated values should, in turn, agree with the values observed in the washings (third column in Table II) which was the case.

State of the catechol amines released on osmotic lysis of amine granules

The amines in water lysates obtained from amine granules were found to be dialysable. The rate was slower than that observed for pure adrenaline hydrochloride used in the same concentration and at the same pH (about 6.5). When small amounts of a low-molecular weight electrolyte (sodium acetate, potassium chloride) were added, the rates for the amines in lysates and for pure adrenaline were similar, however. Experiments with adrenaline adenosinetriphosphate confirmed the suspicion that the slower dialysis of the lysate amine was probably due to the fact that it was only with difficulty that the large triphosphate ion penetrated the membranes used.

On ultrafiltration the amines in water lysates were readily forced through a collodion membrane with the water, whereas the proteins were retained.

The amines in lysates were further found to be taken up on both strong and weak (carboxylic) cation exchangers in the hydrogen form. On paper electrophoresis at pH 6 to 7.5 they moved as a single band, quite independent of the proteins and with the same mobilities as pure adrenaline.

The experiments thus indicate that the stored catechol amines when released by osmotic lysis of the granules occur as ions in true solution.

Discussion

Several observations suggest that at least some of the biogenic amines are stored in the tissues in a similar way as that of the catechol amines in the adrenal medulla. Thus the adrenergic transmitter, noradrenaline, in the autonomic nerves is bound to granules with properties at least partly similar to those of the amine granules in the adrenal medulla (Euler and Hillarp 1956, Euler 1958) and having a content of ATP that is nearly equivalent to the amine (Schümann 1958). The presence of large amounts of ATP in the blood platelets which serve as depots for 5-hydroxytryptamine (5-HT) suggests this nucleotide to be of importance for the storage of this amine, too (Born 1956, Born, Ingram and Stacey 1956, Carlsson and Hillarp 1956). The general depletion by reserpine of the body stores of 5-HT, the adrenergic transmitter and the amines in the adrenal medulla and especially the apparently similar mode of action of this drug on the different amines further suggest a similar storage mechanism (Carlsson *et al.* 1957).

Studies on the properties of the isolated amine granules from the adrenal medulla once suggested that the catechol amines were kept within a semipermeable membrane (HILLARP and NILSON 1954 a). The presence of a granule membrane has been confirmed by electron microscopy (LEVER 1955, SJÖSTRAND and WETZSTEIN 1956, WETZSTEIN 1957) but the morphological studies do not give any clue to the storage problem. It has been claimed that the inner part of the granules have structures in the form of very small granules (SJÖSTRAND and WETZSTEIN 1956), but these may be precipitates produced from osmic acid in the fixation solution on the strong reduction by the amines.

HUGHES, SHORE and BRODIE (1958) have suggested that 5-HT is held in the platelets by an active transport mechanism maintaining the amines against a concentration gradient. Though this hypothesis provides a good explanation for some experimental observations, no palpable evidence is as yet available. Moreover, it is based in part on assumptions concerning the permeability of the platelet membrane, and these assumptions need not be valid either. There is good evidence that the amines in the medullary granules cannot be kept in this way. The fact that the isolated granules may be kept in isotonic media at 0° without any energy supply for several days without any significant release of amines or break-down of ATP and ADP (FALCK, HILLARP and HÖGBERG 1956) is sufficient to disprove any assumption of an active transport mechanism playing any essential role in the maintenance of the very high concentration gradient. Such a mechanism may be involved during the actual accumulation of the amines in the living cell, but all experimental findings (see below) indicate that the accumulated amines must be kept within the granules by other means.

The experiments in this and previous studies strongly support the view that the amines must be stored in a bound state in the medullary granules. The concentrations of amines and adenosinephosphates in the intragranular water — if present as free ions or molecules — can be calculated from the corrected data in Table I to be at least 0.55 M and 0.13 M, respectively. The granules should thus contain an osmolar concentration of solutes more than twice that of blood. Although the activities of the ions is probably low at such concentrations, it does not seem possible that water uptake should not occur. Since the osmotic pressure of the intragranular fluid appears to be maintained by the same constituents as in the external media, it appears justified to conclude that the amines exist in the granules in an osmotically inactive form (CARLSSON and HILLARP 1958). The granule membrane seems freely permeable to adrenaline in the external medium, and added adrenaline passes freely into the intragranular water space and out again on washing of the granules, but no significant exchange between the stored and added adrenaline occurred in spite of favourable conditions. This shows that at most a minor portion of the amines can be present in a free form in the intragranular water and thus indicates that the amines are bound in a non-diffusible state within the granules.

From the determinations of the general chemical composition of the granules an information was obtained which seems of high significance for the understanding of the way in which the amines are bound. The determinations showed that amines, adenosinephosphates, proteins and lipids represent practically the entire content of solids in the granules. It therefore seems improbable for purely quantitative reasons that any other constituent should be directly responsible for the bound state of the amines. There is good evidence that the amines are stored together with equivalent amounts of adenosinephosphates (cf. HILLARP 1958 a, HILLARP and THIEME 1959). Though the adenosinephosphates are well known for their property to form complexes with cations it does not seem likely (see below) that the amines are bound by a complex formation only with these phosphates. It seems necessary to postulate that at least a third component is involved. There is at least suggestive evidence that the intragranular proteins may be the third element (HILLARP 1958 b). This protein fraction, which is released on osmotic lysis, represents almost 80 % of the total granule proteins and contains one main component with a low isoelectric point.

The molecular structure supposed to hold the amines in a non-diffusible state shows in one respect a high stability since undamaged granules may be kept at 0° for considerable periods of time without amine release. From other points of view it is a highly labile structure. Thus a rapid "spontaneous" liberation of equivalent amounts of amines and ATP is elicited on raising of the temperature above + 20° (HILLARP 1958 d). The fact that the intragranular proteins are not released and that the temperature dependance of the reaction is high may suggest an enzymatic attack on the binding site. Apparently the molecular complex is immediately destroyed or at least rapidly broken down on osmotic lysis of the granules, since the amines seem to exist as free ions in the lysate. The destruction of the supposed molecular complex on such a mild treatment must be an important clue to the interpretation of the structure of the amine complex and what forces are responsible for the binding. At present the only permissible conclusions are, however, that no *stable* amine complex exists and that the supposed complex hardly can be built up of amines and adenosinephosphates only.

There are interesting similarities between the findings concerning the amine storage and those found by GALE and coworkers (cf. GALE 1954) for glutamic acid accumulation in *Staphylococcus aureus*. These bacteria are able to accumulate glutamic acid in high concentrations. When released in different ways the accumulated acid does not differ from free glutamic acid, but within the bacterial cells it is kept in a non-diffusible form not exchanging with labelled glutamic acid in spite of a free exchange between glutamic acid of the external medium and of the 'water space' in the cells. Even small monovalent cations such as the potassium ion may be accumulated in a non-exchangeable form in some bacteria (ROBERTS and ROBERTS 1950).

Though experimental findings support the view that the catechol amines are stored in a bound form they also, however, seem to show that the amines exist as free ions when released with the most gentle treatment possible. As long as no definite explanation for this apparent contradiction has been found it seems advisable to have some doubts as to the conclusiveness of the evidence for the bound state of the amines.

Summary

1. When amine granules from cow adrenal medulla were incubated in media containing tritium labelled adrenaline no significant exchange between this adrenaline and the amines stored in the granules occurred in spite of the fact that adrenaline in the external medium freely passes into the intragranular water.

2. The findings in this and previous studies indicate that the stored amines cannot exist in a free form in the intragranular water but are bound in a non-diffusible state in combination with adenosinephosphates and probably with at least a third component.

3. Determinations of the general chemical composition of the granules showed that water (68.5 %), amines (6.7 %), adenosinephosphates (4.5 %), proteins (11.5 %) and lipids (7 %) constitute about 98 % of the granule weight. For quantitative reasons it thus seems improbable that any other constituent should be directly responsible for the bound state of the amines. There is some evidence for the view that the intragranular proteins are a third component in the amine complex.

4. When the stored amines are released from the granules with the most gentle treatment possible (osmotic lysis) they seem to exist as free ions as judged from experiments with dialysis, ultrafiltration, paper ionophoresis and cation exchangers. The supposed molecular complex binding the amines in the granules thus seems to be a very labile complex, which is destroyed on disruption of the granule membrane.

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Enzymic Properties of Cholinesterases in Subcellular Fractions From Rat Brain

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Abstract

HOLMSTEDT, B. and G. TOSCHI. Enzymic properties of cholinesterase in subcellular fractions from rat brain. *Acta physiol. scand.* 1959. *47.* 280—283. — Mitochondria, microsomes and a soluble fraction, prepared from rat brain homogenate by differential centrifugation, were assayed for cholinesterase (ChE) activity with different substrates. The activity of true ChE is higher in mitochondria and microsomes than in the whole homogenate, whereas pseudo-ChE activity is more concentrated in the soluble fraction. The association of true ChE with membrane-rich fractions is stressed.

The cytological location of cholinesterase (ChE) has been investigated by several authors in different ways (GIACOBINI 1959 *a*, *b*, KOELLE 1951, NATHAN and APRISON 1955, TOSCHI 1959). ChE appears to be located mainly in the cytoplasm of neurones, the highest concentration being found in those cell structures which form the microsomal and mitochondrial fractions (NATHAN and APRISON 1955, TOSCHI 1959).

Histochemical investigations, both qualitative and quantitative (GIACOBINI, 1959 *b*, KOELLE 1954), have described the histological distribution of true and pseudo ChE in the nervous tissue, indicating that true ChE is mostly found in neurones and only pseudo ChE in glia and vessels.

Table I. Hydrolysis of different substrates by subcellular fractions of rat brain, compared with the whole homogenate. Activity is determined by electrometric method according to Tammelin, with automatic recording of 6 samples of the same enzyme preparation. Tests run in duplicate with 3 substrates (0.01M ACh, 0.03M BuCh, 0.03M MeCh). Medium: Michel's buffer, pH 8. Activity is expressed as μmoles of acetic acid/ml/min. (A total of 15 experiments is reported.) Each experiment is carried out with a new preparation. DW, preparation treated with distilled water, frozen and thawed. S, submitted to sonic disruption.

Experiment no.	Preparation	Mode of disruption	Protein mg/ml	Activity with BuCh	Activity Ratio ACh/ BuCh	Activity Ratio MeCh/ BuCh
1	Homogenate	DW	3.2	22	8.4	3.6
2	»	S	1.3	7.5	7.5	3.7
3	»	S	1.9	8.5	7.7	3.5
4	»	S	2.6	11	9	3.8
5	Mitochondria	DW	2.3	7.5	14.6	7.6
6	»	DW	1.8	6.5	9.6	4.5
7	»	S	4.5	6	13.2	5.7
8	»	S	4.2	7	11.8	5.4
9	Microsomes	DW	1.7	20	10	4.4
10	»	DW	1.7	19	—	4.6
11	»	S	2.9	16	12	5.7
12	»	S	2.8	22	9.6	4.4
13	Soluble fraction	—	2.5	6	5.6	2.7
14	»	—	2.9	10	5.7	2.7
15	»	—	6	40	3.4	1.9

In the present study the enzymic characteristics of ChE of different subcellular fractions of brain tissue have been investigated with the aim of following the distribution of true and pseudo ChE, as a possible indication of the physiological significance of these enzymes.

Methods

Subcellular fractions from rat brain were obtained by differential centrifugation of homogenates in 0.25 M sucrose. The mitochondrial fraction was prepared according to Brody and Bain (1952). Microsomes were obtained by the quick procedure previously reported (Toschi 1959) (centrifugation for 1 hr. at 105,000 × *g* of the supernatant from centrifugation at 18,000 × *g*). The final supernatant from the microsomal fraction is designated the "soluble fraction". Because of the heterogeneity of the "nuclear" fraction, containing large amounts of cell débris, clumps of cytoplasmic structures, myelin sheaths, red cells and blood vessels (Hanzon and Toschi 1959), this fraction was not examined, but the whole homogenate was used for comparison with the cytoplasmic structures.

Before enzymic assay, the preparations were submitted to various treatments designed to improve the accuracy of the determinations. Complete disruption of the

structures was attempted in order both to expose all the enzyme to the medium and to liberate constituents which can interfere with the hydrolysis and its measurement. As indicated in Table I, one part of the preparations was suspended in distilled water and frozen, then thawed immediately before the assay, rehomogenized and neutralized; another part was submitted to more complete disruption by sonic treatment and removal of dialysable constituents. Suspensions in distilled water were treated in the "Raytheon sonic oscillator" (10 Kilocycles per second) for 30 min, at 4° to 8°; a marked clarification of the suspensions was observed. They were then dialysed overnight at 4° against water (containing a slight amount of Michel's buffer to neutralize the acidity, particularly of mitochondrial fractions), frozen and used as above. The soluble fraction was brought up to proper concentrations of protein and ChE by ultrafiltration in the cold.

The enzymic characteristics of the preparations were outlined by the pattern of activity towards different substrates.

The activity was determined by an electrometric method according to TAMMELIN (1953) with automatic recording of the change in pH. Duplicate samples were run in parallel with three different substrates. The final medium contained Michel's buffer, pH 8 and enzyme preparations at various protein concentration, as shown in Table I. Substrates were acetylcholine iodide (ACh), butyrylcholine iodide (BuCh) and acetyl-β-methylcholine iodide (MeCh). Routinely 3 ml of enzyme preparation were added to 3 ml of Michel's buffer (TAMMELIN 1953) and 0.6 ml of substrate solution to give different final concentrations of protein (1.3 to 6 mg/ml).

The pH at the beginning of the measurement was 7.7—7.9. The temperature was kept at 25°.

Results and Discussion

As illustrated by Table I, the pattern of activity towards different substrates shown by the whole homogenate of rat brain is rather similar to that reported in previous investigations (DAVISON 1953). The observed ratio of activity ACh/BuCh indicates a low percentage of pseudo ChE, according to DAVISON (1953). The values of the same ratio observed with mitochondrial preparations are definitely higher in all the experiments. Microsomes show a ratio of the same order as mitochondria. Moreover they always have a higher specific activity than any other fraction confirming previous findings (TOSCHI 1959). By contrast, a sharp decrease of the ACh/BuCh ratio is observed in the soluble fraction where pseudo ChE appears to be definitely more concentrated.

The values observed for the activity ratio MeCh/BuCh in all experiments follow the same pattern as that of ACh/BuCh, thus confirming and emphasizing the higher concentration of true ChE in mitochondria and microsomes, compared with the whole homogenate and the soluble fraction. Apparently the sonic treatment did not increase the specific activity of the preparations, but rather reduced it, and had no appreciable effect on the pattern of activity with different substrates.

These results indicate that true ChE is more concentrated in those fractions which are formed more or less completely by membranes (mitochondria and microsomes). Microsomal membranes are supposed to originate mostly

from the cytoplasmic membranes of neurones (HANZON and TOSCHI 1959) and the ChE activity has proved to be firmly associated with them (TOSCHI 1959). The preferential concentration of true ChE on different cell membranes strongly suggests that these are the sites in the cell where this specific enzyme plays its physiological role. By contrast, pseudo ChE is more concentrated in the soluble fraction which is formed both from intracellular and extracellular fluids.

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Distribution of Acetocholinesterase in the Ganglion Cells of Various Sympathetic Ganglia

By

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Abstract

Holmstedt, B. and F. Sjöqvist. Distribution of acetocholinesterase in the ganglion cells of various sympathetic ganglia. *Acta physiol. scand.* 1959. *47*. 284—296. — The purpose of the present investigation has been to find out if differences exist between the acetocholinesterase activity of different ganglia and different ganglion cells. The histochemical study has been carried out with the modified thiocholine method, and control experiments of total cholinesterase activity have been performed with an electrometric technique. Three groups of ganglion cells with respect to staining intensity can be distinguished, group I heavily stained cells, group II moderately to faintly stained cells and group III unstained cells. Group I has been predominantly found in the stellate ganglion (7 %). The total number of stained cells (group I + group II) was: sup.cerv.gangl. 21.3 %, stellate gangl. 14.9 %, sup.mes. ganglion 6.8 % and coeliac gangl. 4.8 %. The electrometric method gave the following values of total acetocholinesterase activity: 71.5 × 10^{-3}, 51.5 × 10^{-3}, 29.5 × 10^{-3} and 30.0 × 10^{-3} μmoles HAc per mg dry weight per min. The differences found between the sup.cerv.gangl. and the stellate gangl. on one hand and the abdominal ganglia on the other are highly significant. The influence of different incubation times has been studied. Different possibilities to explain these histochemical and biochemical results, suggestive of at least a dual type of ganglion cells, are discussed.

Ever since the experiments of KIBJAKOW (1933) the superior cervical ganglion has been extensively used in mammalian physiology. The relative accessability of this ganglion, the possibility to perfuse it and to record the contractions of the nictitating membrane have made it the favourite ganglion for pharmacological experiments (FELDBERG and GADDUM 1934, BROWN and FELDBERG 1936, PERRY 1953, EMMELIN and MACINTOSH 1956). The classical concept of cholinergic synaptic transmission is based primarily on experiments made with the superior cervical ganglion. Less attention has been paid to other sympathetic ganglia, although the generalization of transmission theories has included them as well as ganglia of other kinds and even the central nervous system.

The advent of a reliable histochemical technique for the staining of cholinesterase (KOELLE 1951) allows a morphological study of the distribution of the enzymes responsible for the destruction of acetylcholine. Subsequent modifications of this method, the thiocholine method, allow a more accurate localization of the two groups of enzymes, with less risk of diffusion artefacts (HOLMSTEDT 1957). Histochemical methods offer, provided accurate biochemical controls are performed, the possibility to correlate morphological localization of enzymes with known pharmacological and physiological data.

The purpose of the present investigation has been to find out, if differences in the number of ganglion cells possible to stain for acetocholinesterase activity exist within various sympathetic ganglia in the cat. Control experiments of total cholinesterase activity have been carried out with a biochemical technique.

Material and Methods

Four sympathetic ganglia were studied, the superior cervical ganglion, the stellate ganglion, the coeliac ganglion and the superior mesenteric ganglion. The ganglia were removed from cats of different sexes anesthetized with ether and killed by intravenous injection of air.

Histochemical technique.

After removal of the ganglia they were carefully freed from contaminating tissue and blood, and put air-tight in the refrigerator (— 5° C) as soon as possible (1—1.5 hours) to prevent drying. Fresh frozen sections (10—15 μ thick) were cut the same or the following day, mounted on slides and stained according to the modified thiocholine method (HOLMSTEDT 1957). The four sympathetic ganglia were stained simultaneously and passed the successive jars at the same time. The combinations of substrate and inhibitors used are seen in Table I.

An incubation time of 30 min was uniformly used except in the instances where varying incubation times were studied.

The slides were mounted and viewed without further treatment in the ordinary light microscope and photographed if a permanent record was required. The ganglion cells were counted in a counting chamber. Care was taken to count from one end of the section to the other at different levels (10—15) of the ganglia. To arrive at reliable per-

Table I. Combination of inhibitors and substrate

Slide	Treatment		ChE visualized
	Inhibitor	Substrate	
A	—	AcThCh	AcChE + BuChE
B	Mipafox 4×10^{-6}	AcThCh	AcChE
D	Mipafox 4×10^{-6} + +BW284c51 5×10^{-6}	AcThCh	Blank

AcThCh = acetylthiocholine
AcChE = acetocholinesterase = true cholinesterase
BuChE = butyrocholinesterase = pseudocholinesterase

centages of figures, a large number of ganglion cells was counted, approximately 1,000 per ganglion and cat (Table II). The ganglion cells were arbitrarily divided in three groups according to their intensity of staining (compare results).

It is recognized that subjective estimation may give misleading results due to the difficulty in distinguishing colour intensities especially in group II which presents more differences than the other groups. This was avoided by repeatedly altering the focus at different levels, and by always having the same person count the various cells.

Biochemical technique

The biochemical controls were performed with an electrometric technique, described by TAMMELIN *et al.* (1951, 1952, 1953).

Enzymes

Freeze-dried ganglia were used because of the greater difficulty in homogenizing fresh ganglia in an effective manner. After removal from the cat (when possible bilaterally) the ganglia were decapsulated, the sympathetic nerve was cut adjacently, the ganglia were kept for 0—12 hours in air-tight refrigerator and then freeze-dried and later used as the source of enzyme. The weights before and after freeze-drying were investigated. The medium weight loss was about 75 %. The ganglia were homogenized in Michel's buffer in the dilution 2.95 mg ganglion per ml. A Buehler homogenizer was used during 15 min and the speed was 12,000 r p m. The number of ganglia collected is seen in Table III. The material is divided into two groups collected at different times and thus two estimations were made.

Inhibitor

Mipafox (bismonoisopropylamidophosphoryl fluoride 4×10^{-6} M in final solution) was added to the enzyme exactly 30 min before the addition of the substrate.

Substrate

Acetylcholine iodide, 7.32×10^{-3} M in the final solution.

Buffer

Michel's buffer according to TAMMELIN (1951, 1952 and 1953).

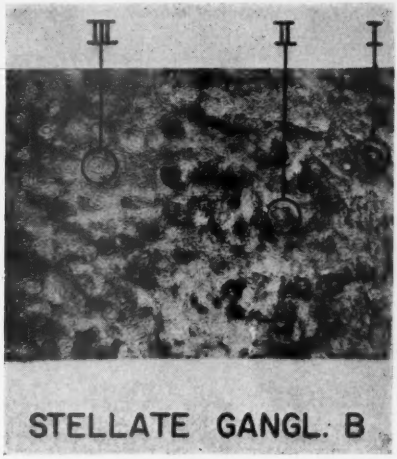

Fig. 1. Definition of the three groups of cells counted in the investigation. Magnification 137 times.

Reaction mixture

The final reaction mixture had the following composition.
Michel's buffer 1.5 ml
Enzyme 1.5 ml
Aq. dest. 3.0 (2.4) ml
Inhibitor 0.6 ml
Substrate 0.6 ml

Measurement of enzyme activity

Enzyme activity was determined at 25° C and pH 8 and expressed as μmoles acetic acid (HAc) liberated per ml reaction mixture per min. The apparatus was calibrated as described by TAMMELIN (1951). These values were later recalculated to μmoles HAc liberated per mg ganglion per min.

Results

Histochemical method. Fig. 1 demonstrates the various types of ganglion cells counted. Group I consists of homogeneously heavily stained cells, easy to distinguish from the surrounding glial tissue. Group II is heterogeneous in staining intensity and includes moderately to faintly stained cells. Group III finally consists of unstained cells. In the present investigation only the ganglion cells have been considered and no attention paid to other localisation of cholinesterase within the ganglia. As seen from Fig. 1 the membranes seem to be somewhat more intensely stained whereas the nuclei are very faintly stained if stained at all.

Fig. 2 shows that the various types of cells are not uniformly distributed. This uneven distribution is most apparent in the stellate ganglion, less evident

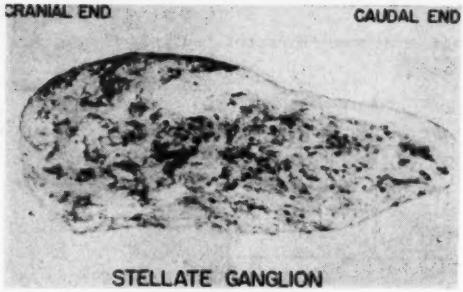

Fig. 2. Survey of histochemically stained ganglion cells in the stellate ganglion. Magnification 22 times.

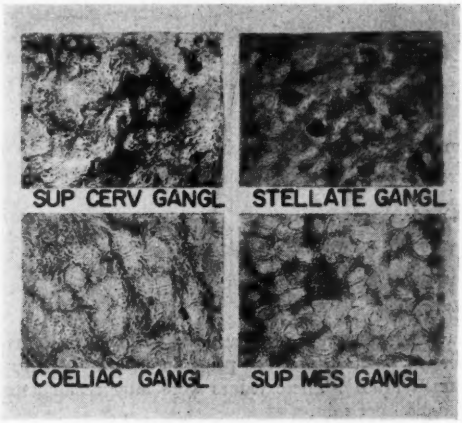

Fig. 3. Histochemical picture of different sympathetic ganglia. Magnification 100 times.

in the others. In the stellate ganglion the heavily stained cells are about 5—10 times as frequent in the caudal as in the cranial end.

Fig. 3 and 4 demonstrate the histochemical pattern of the three groups of cells in the sympathetic ganglia investigated. Fig. 4 gives the number of heavily stained, faintly stained and unstained cells in per cent of the total number of cells counted. The ordinate for the heavily stained cells is purposely a scale 10 times that of the other groups in order to demonstrate the differences between the ganglia. The total number of cells counted per ganglion is around 5,000 and the number of experimental animals 5 per ganglion (See Table II). From Fig. 4 is evident, that the stellate ganglion is especially rich in heavily stained cells (approximately 7 %). The superior cervical ganglion has less

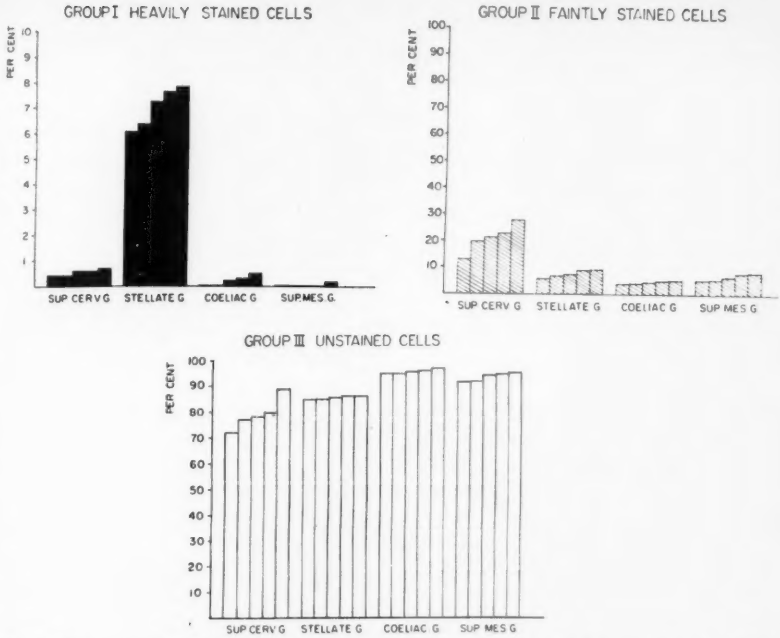

Fig. 4. Histogram of different cell types. Every bar corresponds to one ganglion and one cat and approximately 1,000 cells counted.

than one tenth of this. Statistical analysis (Table III) reveals that the difference is highly significant ($p < 0.001$). It also appears that the discrepancies in group I between the other ganglia are not significant. On the other hand the sup.cerv.gangl. has significantly more stained cells in group II. If all stainable cells (group I + group II) are taken together it is evident, that they are approximately equal in the stellate and sup.cerv.gangl. It also appears that the abdominal ganglia contain considerably fewer such cells and the t-values reveal highly significant deviations. The difference between the coel. and sup. mes.gangl. is almost significant. The best view of these results is the histogram of the number of unstained cells. The total numbers of the stained cells for the different ganglia are as follows: superior cervical ganglion 21.3 %, stellate ganglion 14.9 %, superior mesenteric ganglion 6.8 % and coeliac ganglion 4.8 %. The biggest variation between individual cats was found in group II for the superior cervical ganglion (11—28 %) which seems reasonable because of the difference in staining intensity of these cells. The investigation has not shown any real difference between individual cats, between males and females or between right and left superior cervical and stellate ganglia.

Table II. Total material of ganglion cells

Ganglion	Slide	Numbers of stained cells in per cent. ($\overline{X} \pm$ S. E.)			Total number of cells counted
		Group I	Group II	Group III	
sup.cerv.	A	0.3 ± 0.2	19.3 ± 1.9	80.5 ± 1.8	1,200
sup.cerv.	B	0.5 ± 0.1	20.3 ± 2.7	79.2 ± 2.7	3,967
stellate	A	6.9 ± 0.7	7.6 ± 0.6	85.3 ± 0.2	1,200
stellate	B	7.1 ± 0.4	7.4 ± 0.7	85.7 ± 0.4	6,139
coel.	A	0.1 ± 0.2	3.9 ± 0.6	96.0 ± 0.6	2,403
coel.	B	0.2 ± 0.1	4.4 ± 0.2	95.4 ± 0.3	6,330
sup.mes.	A	0.0	5.2 ± 0.4	94.8 ± 0.4	1,741
sup.mes.	B	0.04 ± 0.04	6.7 ± 0.6	93.2 ± 0.6	4,253

$\overline{X}$ = mean of x.
S. E. = standard error of the mean.

Table III. Test for significance (t-values)

Differences between ganglia in pairs	Group I	Group II	Group III
^{B}stell.−^{B}sup.cerv.	15,9***	− 5.1**	2.4
^{B}stell.−^{B}coel.	18,6***	0.9	− 22.3***
^{B}coel.−^{B}sup.mes.	1.7	− 3.2*	3.3*

t is based on differences between ganglia in pairs of every cat. Group III is included for the sake of completeness. Degrees of freedom = 4.

0.05 > P > 0.01 * almost significant
0.01 > P > 0.001** significant
0.001 > P *** highly significant

The following controls have been included in the investigation (Fig. 5). Slide A gives the upper limit of the number of stained cells. When butyrocholinesterase activity of the glial cells is present the unstained cells appear more outstanding at the same time as the cell borders of the stained cells become more difficult to distinguish and consequently may be underrepresented. Accordingly the percentage of cells in group III in slide B must not significantly exceed that in slide A. Table II shows that this is not the case. This table contains the total material and indirectly confirms, that the ganglion cells do not contain butyrocholinesterase. In that case the number of stained cells

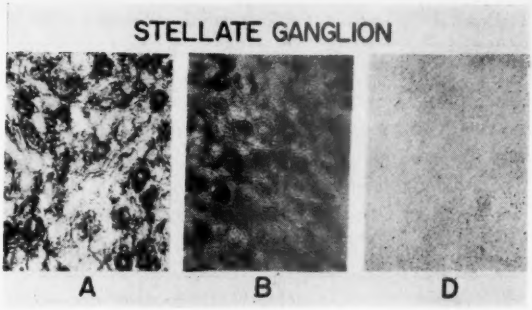

Fig. 5. Histochemical picture at different combinations of substrate and inhibitor according to Table I. Magnification 106 times.

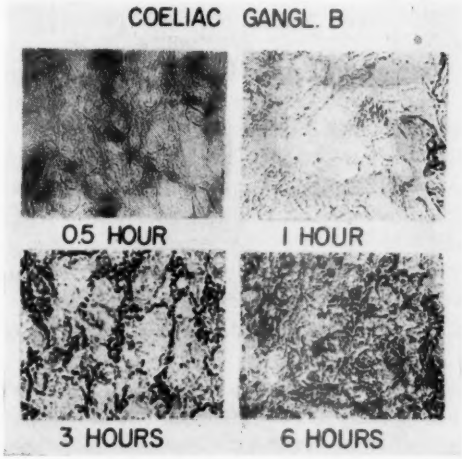

Fig. 6. Histochemical pattern of the coeliac ganglion at various incubation times. Magnification 100 times.

in slide A should have exceeded that in B. Further controls with the specific substrate butyrylthiocholine also confirmed this. Slide D reveals unspecific contamination due to salts in the incubation solution. No D slides were found possible to misinterpret as containing cholinesterase activity.

The influence of variations in incubation time has been considered for all ganglia. Incubation times below 30 min gave lower values for the number of stained cells than those above 30 min. No differences seemed to exist between 30, 40, 50 and 60 min. The influence of very long incubation times up to 6

Table IV. Total ChE- and AcChE-activity of different ganglia, estimated with the biochemical method

Ganglion	Number of gangl.invest.	Inhibitor.	ChE-activity of ganglia in μmoles HAc per mg per min. $\times 10^{-3}$.	
			Freeze-dried ganglia	Freeze-dried ganglia, values recalculated to wet weight
sup.cerv.	6	—	122.7	30.7
	14		113.9	28.5
	6	Mipafox	75.2	18.8
	14		68.3	17.1
stellate	6	—	105.7	26.4
	17		110.8	27.7
	6	Mipafox	47.1	11.8
	17		56.0	14.0
coel.	10	—	84.7	21.2
	7	Mipafox	25.8	6.4
	10		34.0	8.5
sup.mes.	7	—	75.3	18.4
	6		61.4	15.4
	7	Mipafox	32.0	8.0
	6		26.7	6.7

hours was studied in the coeliac ganglion, which ganglion has the lowest number of stained cells. No significant differences existed between the incubation times 0.5—3 hours. From Fig. 6 it can be seen that at 6 hours the staining intensity is considerably increased but that unstained cell bodies still remain.

Biochemical method

The biochemical control experiments are presented in Table IV. From the table it can be seen that the two cranial ganglia have about twice the AcChE-activity of the abdominal ganglia. The relative proportions of the AcChE-content in different ganglia are the same as in the histochemical method. Differences in total ChE-activity exist between the cervical and stellate ganglion on the one hand and the abdominal ganglia on the other. No appreciable differences were found to exist between the right and left side of the sup.cerv. and stellate ganglia.

Discussion

In the present investigation two methods of estimating ChE-activity have been used. The interest has been primarily to study the AcChE of the ganglion cells. Controls have been included with the purpose of ascertaining that the correct type of ChE was visualized and also to facilitate the counting of unstained cells (Table I). The use of a selective inhibitor (Mipafox) proves, that the nerve cells from the sympathetic ganglia of the cat studied contain only AcChE as previously described by KOELLE (1951) and HOLMSTEDT (1957). BuChE-activity was never seen in the ganglion cells of this species but has been demonstrated in the rat by KOELLE (1954).

The main interest was to see, if the modified histochemical technique could conclusively demonstrate differences in cholinesterase activity in the cells of different sympathetic ganglia. Not all the ganglion cells showed a measurable cholinesterase activity in contrast to the cells of the spinal cord studied by GIACOBINI and HOLMSTEDT (1958). The relatively large number of unstained cells is clearly demonstrated in the lower part of Fig. 4. It is, thus, apparent that relatively few cells of the sympathetic ganglia are "cholinergic" in the sense that they contain cholinesterase.

If an approximation of the cells into three groups is made, group I significantly differs from the other cells, not only because of the staining intensity but also because of the homogeneous staining. Group III consists of well defined unstained cells. By contrast group II contains very heterogeneous cells which have been arbitrarily counted together without further division into subgroups. No such subdivision can be reasonably made within the errors of the method. On the other hand, a reason for the validity of the method is, that the three groups of cells are distinguishable over a wide range of incubation times. The shortest one used in the present investigation was 10 min and the longest 6 hours. The difference between the various ganglia studied is independent of the incubation time, although the intensity of the different cells increases with increased incubation time. Even after 6 hours the unstained cells are still visible in the ganglia (the coeliac ganglion being used due to its relatively large number of unstained cells).

The finding of unstained cells even after prolonged incubation times is in contrast to the results of KOELLE (1955) where ten micra sections of the sup. cerv. ganglion were incubated for 2 hours. The author states, that a small number of neurons was heavily stained and the remainder were stained moderately or lightly. An explanation of the discrepancies of the two investigations could be the omission of ammonium sulfide as well as the different incubation times. In any event the differences between the various ganglia remain at increasing incubation times. The present results are in conformity with those of GIACOBINI (1957) who used a micro diver technique and frog ganglia and found a certain number of cells where no activity could be demonstrated

even after 8 hours of incubation. This was approximated to a 24 hour incubation in the histochemical incubation solution.

The histochemical method used by the present authors seems to be a reasonable tool for the semi-quantitative judgment of cholinesterase activity of the sympathetic ganglia. Further confirmation of the levels of enzyme activity has been obtained by cholinesterase determinations in freeze-dried sympathetic ganglia.

With regard to acetocholinesterase activity the same relationship between the different ganglia could be demonstrated with the biochemical technique. The sup.cervical ganglion seems to have slightly more activity than the stellate ganglion exactly as found histochemically where the total number of stained cells was higher in the former ganglion. The two abdominal ganglia show about the half of the activity of the two cranial ganglia and are approximately equal. It must be stressed that the activities presented in Table III refer to the total acetocholinesterase activity. Thus, other parts of the ganglion than the ganglion cells, such as neurites and dendrites, are included whereas the figures for the histochemical determination are referable to the acetocholinesterase activity of the ganglion cells alone. However, the biochemical results show that considerable differences exist between individual ganglia. Of importance is the fact, that to avoid prejudice our biochemical controls were made after publishing of the histochemical results in a preliminary report (HOLMSTEDT and SJÖQVIST 1957).

Few experiments concerning acetocholinesterase activity have been made before. MCLENNAN (1954) investigated the total cholinesterase content of normal and axotomized ganglia of the rat. According to this investigation the rat ganglion contains considerably more ChE than that of the cat. Similar results have been obtained by BROWN (1958) and DAHR (1958). HOLADAY, KAMIJO and KOELLE (1954) found a high percentage of stained ganglion cells in the sup.cerv. ganglion of rats. The rat ganglion is also less susceptible to acetylcholine than that of the cat (KALLER 1956).

The cells of various staining intensities found in the present investigation call to mind previous pharmacological and physiological experiments indicating functional differences in ganglion cells. Thus, SHAW (1950) in a series of papers demonstrated a quantitative difference between various cells in the sup.cerv. ganglion with regard to their susceptibility to tetraethylammonium, curare and pentamethonium. Certain experiments suggestive of inhibitory neurones in the superior cervical ganglion of the turtle have been presented by LORENTE DE NÓ and LAPORTE (1950). Indications of the presence of such neurons in mammals is lacking. The inhibitory substance isolated from mammalian brain by FLOREY and MCLENNAN (1955) and the activity of which is at least partly thought to be due to γ-aminobutyric acid (MCLENNAN 1957) has also been reported to affect transmission in various sympathetic ganglia differently. This may, however, be due to the local application and awaits further elucida-

tion. Even when active substances are applied to the sup.cerv. ganglion by perfusion considerable individual differences exist (AMBACHE 1956) and a dual type of transmission is conceivable (KEWITZ 1950). The postganglionic cholinergic sympathetic fibres such as the vasodilator fibres demonstrated by UVNÄS and FOLKOW (1948) may also bear a certain relationship to the types of cells demonstrated. With regard to the small number of stained cells and the low total ChE-activity of the abdominal ganglia it is interesting to note that the peripheral circulation of the intestine is particularly susceptible to ChE-inhibitors (DALY and WRIGHT 1956).

The above-mentioned functional studies suggestive of at least a dual type of ganglion cells are, of course, not the only ones possible to refer to the types of cells found histochemically. Other possibilities exist, such as different stages in the synthesis of the enzyme, but the latter is less likely because of the constant findings of the different cell groups in various ganglia. Whatever the explanation may be, the present investigation adds weight to the conception that the cell population of various sympathetic ganglia is pharmacologically and physiologically unhomogeneous. The findings are worth bearing in mind whenever too far-reaching conclusions regarding transmission are drawn from experiments on one ganglion.

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Nociceptive Reflexes of the Human Foot
The Plantar Responses[1]

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The significance of the normal plantar response of the human foot and its relation to the pathological plantar response is still obscure. In the present investigation the reflexes have been studied electromyographically. Our observations are of interest because they show in the intact organism the functional organization of a defence reaction, extensively investigated by SHERRINGTON and others in spinal and decerebrate animals.

It was found that electric shock stimulation of the skin of the foot produces widely different reflex patterns, according to the site of stimulation. A stimulus delivered to the ball or mid-portion of the sole of the foot evokes contraction of the short toe flexors and a reciprocal inhibition of the antagonistic short extensors. Thus, the toes are plantar flexed (physiological extension). Simultaneously there is contraction of the flexors at the ankle, knee and hip joints and reciprocal inhibition of the extensors. These are the movements of the normal plantar response as seen by the clinician.

A shift of the stimulus to the plantar surface of the toes radically changes the reflex pattern. The toes are now dorsiflexed but the movements at the other joints remain unchanged. A stimulus to the plantar surface of the heel again changes the reflex pattern, now producing extension instead of dorsiflexion at the ankle. A stimulus to the dorsal surface of the foot may evoke a weak contraction of the gastrocnemius with resultant extension of the foot. The flexion at the knee, however, is the main reflex movement.

Thus it appears that each skin area of the foot is functionally associated with the appropriate withdrawal pattern. A general flexion reflex and more local

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extension reflexes are integrated in a movement producing withdrawal from the stimulus regardless of the site of stimulation. Since a similar mechanism has been observed in investigations of the skin reflexes of the trunk (KUGELBERG and HAGBARTH 1958), it may be inferred that this is a general principle of functional organisation of nociceptive reflexes in man.

The normal reflex pattern observed may, however, be more or less broken down when the reflex centre is deprived of suprasegmental control. The extensor reflexes are abolished and a general flexion of the entire limb dominates, regardless of the site of stimulation. It has been thought that this flexion reflex is not present in the normal state (*e. g.* WALSHE 1914). Preliminary observations on the Babinski reflex suggest that the pathological reflex pattern is the result of an imbalance between the extensor and flexor reflexes observed in the normal state, with predominance of the flexor reflex in a greater part of the receptive field.

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